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## *DIPETALONEMA ARBUTA* N. SP. (NEMATODA) FROM THE PORCUPINE, *ERETHIZON DORSATUM* (L.)

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The filarioid nematode commonly found in the peritoneal cavity of *Erethizon dorsatum* (L.) has been identified by Canavan (1929 and 1931) and Jellison (1933) as *Dipetalonema diacantha* (Molin, 1858). It was first identified as *Filaria diacantha* Molin, 1858 by Boulenger (1920) who referred it to the genus *Acanthocheilonema* Cobbold, 1870 as emended by Railliet, Henry, and Langeron (1912). This genus was considered to be a synonym of *Dipetalonema* Diesing, 1861 by Yorke and Maplestone (1926).

*F. diacantha* recently has been redescribed and made the type of a new genus, *Molinema* Teixeira de Freitas and Lent, 1939. Specimens from *E. dorsatum* available to me do not agree morphologically with *M. diacantha* as redescribed from *Coendou villosus* (Cuv.) by these authors. Since specimens from this host or from the type host of Molin's species, *Coendou prehensilis,* are not available to me I must accept the Brazilian workers' redescription of the species objectively and must conclude that the specimens from *E. dorsatum* represent a previously unrecognized species of *Dipetalonema;* for it the name *D. arbuta* is proposed.

*Dipetalonema arbuta* n. sp.

(FIGS. 1–7)

*Description: Dipetalonema:* Oral opening round; 8 cephalic papillae; amphids lateral. Ventrolateral terminal caudal conical processes prominent in both sexes.

*Male* 22 to 37 mm (31 mm)[2] long by 0.17 to 0.21 mm wide. Esophagus 2.20 to 2.72 mm (2.39 mm) long; anterior portion 0.39 to 0.49 mm (0.43 mm) long by 0.025 to 0.037 mm (0.032 mm) maximum width; posterior portion 1.83 to 2.23 mm (1.98 mm) long by 0.043 to 0.070 mm (0.055 mm) maximum width. Nerve ring 0.20 to 0.24 mm from anterior extremity. Cloacal aperture 0.23 to 0.33 mm (0.27 mm) from caudal extremity. Pendunculated genital papillae 7 pairs, 4 preanal and 3 postanal, and additional medioventral double one between the 3rd and 4th preanals, the last preanals in adanal position, the last postanals 0.016 to 0.025 mm from caudal extremity. Long spicule 0.212 to 0.225 mm (0.220 mm), anterior portion tubular, posterior filiform, hooked. Short spicule 0.112 to 0.125 mm (0.119 mm), stout, tubular, bent in middle, enlarged posterior portion, hooked. Ratio of spicules 1:1.8 to 1:2. Gubernaculum absent. Phasmids present. Posterior portion of body spiralled in 4 to 6 loops. Longitudinally striated transverse ridges on ventral side in coiled region of body anterior to cloacal aperture, 0.023 to 0.029 mm apart anteriorly, 0.008 to 0.012 mm apart posteriorly.

*Female* 58 to 82 mm (68 mm) long by 0.25 to 0.33 mm (0.27 mm) maximum width. Esophagus 2.07 to 2.77 mm (2.35 mm) long; anterior portion 0.28 to 0.48 mm (0.34 mm) long by 0.035 to 0.047 mm (0.042 mm) maximum width; posterior portion 1.77 to 2.29 mm (2.01 mm) long by 0.064 to 0.082 mm (0.072 mm) maximum width. Nerve ring 0.19 to 0.26 mm (0.23 mm) from

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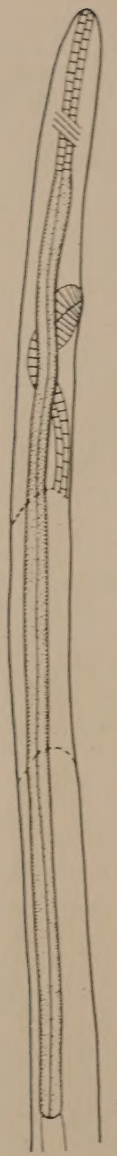
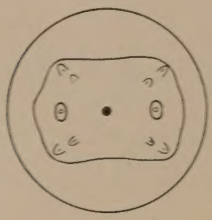
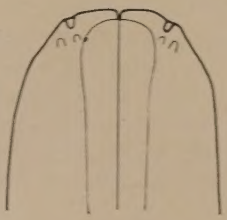
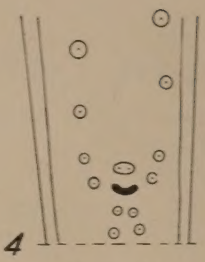
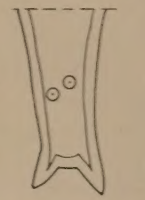
[2] The figures in ( ) represent average values.

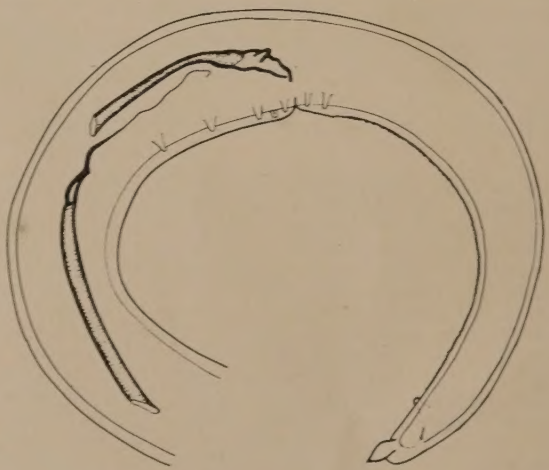


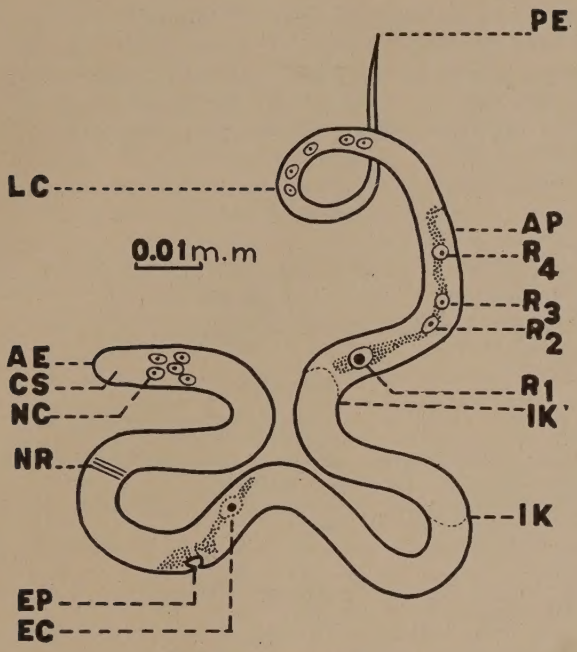




All figures were drawn with the aid of a camera lucida.

FIGS. 1–3. Adult female: 1, anterior end, lateral view; 2, anterior extremity, en face view; 3, anterior extremity, dorsal view.

FIGS. 4–6. Adult male: 4, genital papillae of cloacal region, ventral view; 5, tip of tail, ventral view; 6, caudal region, lateral view.




*Dipetalonema arbuta* n. sp.

FIG. 7. Microfilaria of *Dipetalonema arbuta* n. sp., drawn with aid of camera lucida.

AE—anterior extremity
AP—anal pore
CS—cephalic space
EC—excretory cell
EP—excretory pore
IK—anterior end of "Innen Körper"
IK'—posterior end of "Innen Körper"
LC—last cell of nuclear column
NC—first cells of nuclear column
NR—nerve ring
PE—posterior extremity
$R_1$–$R_4$—1st, 2nd, 3rd, and 4th rectal cells

anterior extremity. Anus 0.20 to 0.25 mm from caudal extremity. Phasmids present. Vulva 0.45 to 0.85 mm (0.70 mm) from anterior extremity.

*Microfilariae* not sheathed, 0.280 to 0.297 mm (0.288 mm) long by 0.006 mm wide (20 specimens measured in aqueous Azur II). Head obtuse. Body slender. Tail long, slender, and pointed. Cephalic space longer than its width. "Innen Körper" begins 132 to 155 μ from the anterior extremity and continues to the anterior border of the first rectal cell. Second and third rectal cells midway between the first and fourth. Fourth rectal cell just anterior to anal pore. Last nucleus of the nuclear column about midway between anal pore and posterior extremity. Last 4 to 6 nuclei typically arranged in tandem, attenuated, and scattered with spaces between. Position of anatomical points in terms of distance from the anterior extremity expressed in percentages of the worm's total length (av. of 20 specimens): length of cephalic space 2.3 per cent, width of cephalic space 1.7 per cent, nerve ring 20.4 per cent, excretory pore 27.1 per cent, excretory cell 31.9 per cent, first rectal cell 65.0 per cent, anal pore 74.5 per cent.

*Host: Erethizon dorsatum* (L.).

*Location:* Adults in pleural, peritoneal and pericardial cavities. Microfilariae in blood and body fluids.

*Locality:* Northern Minnesota, U. S. A.

*Type specimens:* Holotype male, U. S. N. M. Helm. Coll. No. 45037; paratypes, 3 males, 3 females, and dissections, No. 45038. Other paratypes in the Department of Zoology, University of Minnesota.

*Remarks:* 6 male and 6 female adults were measured for the above descriptions except for body length for which 10 males and 20 females were measured.

*D. arbuta* may be distinguished from *M. diacantha* by certain differences. *M. diacantha* has 2 lips with 6 papillae, *D. arbuta* has no lips but has 8 cephalic papillae. The males of *M. diacantha* are from 1⅓ to 2 times as long as *D. arbuta* males, but the females of the former are only slightly larger. In *M. diacantha* the vulva is 0.83 to 1.33 mm from the anterior end, in *D. arbuta,* 0.45 to 0.85 mm. The ratio of the spicule lengths in *M. diacantha* is 1 : 1.3 to 1 : 1.4, in *D. arbuta,* 1 : 1.8 to 1 : 2. *M. diacantha* has 4 pair of preanal and 1 pair of postanal papillae, *D. arbuta* has 4 preanal pair, 1 median double preanal, and 3 postanal pair.

The microfilariae of *M. diacantha* are 0.20 to 0.23 mm long and 8 μ wide, sheathed and with obtuse extremities; those of *D. arbuta,* 0.280 to 0.297 mm long and 6 μ wide, *unsheathed* and with a long pointed tail.

The development in various species of mosquitoes has been traced and will be reported later.

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# MOSQUITO VECTORS AND LARVAL DEVELOPMENT OF *DIPETALONEMA ARBUTA* HIGHBY (NEMATODA) FROM THE PORCUPINE, *ERETHIZON DORSATUM*

PAUL R. HIGHBY

The porcupine, *Erethizon dorsatum* (L.) in Minnesota is commonly parasitized with *Dipetalonema arbuta* Highby, 1943 in its peritoneal cavity and *Dirofilaria spinosa* Canavan, 1929 in its dorsal subcutaneous connective tissue. These worms were previously reported from this host by Boulenger (1920), Canavan (1929 and 1931) and Jellison (1933). I examined 28 porcupines from northern Minnesota and found 10 infected with both species, 2 with *D. spinosa* only, 10 with *Dipetalonema arbuta* only, and 6 with no filarioids.

Microfilariae of these filarioids may be found in the infected porcupine's blood, both peripheral and central, the pericardial, pleural and peritoneal fluids, the urine, the lungs and liver. The microfilariae of *D. arbuta* were recovered also from the mixed amniotic fluid and blood of a fetus removed from an infected porcupine.

*Microfilaria arbuta* may be distinguished from *Mf. spinosa* in both wet and dry preparations by three characters:—*Mf. arbuta* has a cephalic space which is longer than its width while that of *Mf. spinosa* is shorter than its width; the terminal nuclei at the posterior end of the nuclear column of *Mf. arbuta* present a more scattered appearance than the more compact arrangement seen in *Mf. spinosa;* and in *Mf. arbuta* an "Innen Körper" is present, but none is apparent in *Mf. spinosa*. *Mf. arbuta* was described in detail by Highby (1943).

## EXPERIMENTS TO DETERMINE SUSCEPTIBLE VECTORS

Feeding experiments were attempted with laboratory-reared native mosquitoes and simulids without success for the insects failed to feed. However, an exotic species, *Aedes aegypti*, reared from eggs imported from Florida fed avidly on the porcupine, and in this species development of *Dipetalonema arbuta* was completed.

Wild adult mosquitoes were used in further feeding experiments. Of 337 mosquitoes dissected after feeding upon porcupines infected with *D. arbuta* 52 per cent became infected. That these infections were contracted from the experimental source of infection may be inferred by comparison with the natural incidence of filariasis in wild mosquitoes from this locality.

A series of dissections of captured local mosquitoes experimentally fed was made in connection with this and subsequent experiments. These experimental mosquitoes represented 12 species of demonstrated susceptibility to filarioid infections. Nine of the 12 species had been shown to be susceptible to the development of *Dirofilaria immitis* by Yen (1938). Specimens of three other species were also used experimentally of which two were shown to be susceptible and one probably susceptible to *D. arbuta* in this study. In addition specimens of 11 other species were examined, but since their susceptibilities were not demonstrated they are

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not included in the calculation of the natural incidence of filariasis. In 340 mosquitoes dissected before the 9th day after feeding, the minimum incubation period required for development of the 3rd larval stage of *D. arbuta,* no precociously developed larval stages were found. This would indicate a natural incidence of "no filariasis" in 340 local wild mosquitoes and, by inference, that the 52 per cent infection of my experimental mosquitoes was contracted from the infected porcupine.

### *Susceptibility of Mosquitoes to* D. arbuta *Development*

Mosquitoes were considered to be susceptible only when the infective (3rd larval) stage was recovered following its feeding on the infected host. The method of the experiment was in itself selective in that the mosquitoes collected were species attacking man. Further selection occurred in the feeding cage for many species would not feed on the porcupine.

In laboratory-reared *Aedes aegypti,* a species foreign to Minnesota, the embryos of *D. arbuta* developed into the infective stage in 10 days. Of 14 mosquitoes which survived the 10-day incubation period 12 yielded the infective stage of the worm.

In *A. canadensis* Theobald the *D. arbuta* embryos developed into the infective stage in 9 days. From all of 15 mosquitoes which survived the incubation period the infective stage was recovered. One mosquito yielded 20 infective stages.

In *A. cinereus* Meigen complete development of *D. arbuta* occurred on the 9th day. Infective stages were recovered from 17 of the 19 *A. cinereus* which survived the incubation period. As many as 29 infective stages were found in one mosquito. From another mosquito 129 first stage larvae were recovered on the 5th day of incubation.

In *A. excrucians* Walker no infective stage larvae were obtained but the recovery from one mosquito of 70 live second stage larvae on the 9th day indicated the probable susceptibility of this species. In another, 121 first stage larvae were found on the 3rd day.

In *A. fitchii* Felt and Young the larvae of *D. arbuta* completed its development on the 10th day of incubation. All of four specimens which survived the incubation period yielded infective stages. From one mosquito 66 second stage larvae were recovered on the 8th day of incubation.

From *A. stimulans* Walker the infective stage was recovered on the 10th day of incubation. All of 8 specimens which lived longer than 10 days yielded infective stages. From one mosquito 28 infective stages were recovered.

Of 28 specimens of *A. vexans* Meigen dissected between the 11th and 61st days after the experimental feeding only one had the infective stage larva although all 28 were infected with less developed forms. In this particular specimen after 29 days of incubation 33 parasites were recovered of which 9 were encapsulated, 4 infective stages (1 encapsulated), 17 second larval stages, and 12 first larval stages (8 encapsulated). In this species a higher incidence of encapsulation of the parasite was associated with resistance to and retardation of its development.

From *Taeniorhynchus perturbans* Walker, out of 9 engorged specimens two were infected, of which one yielded 5 infective stage larvae after 15 days of incubation.

All the above named mosquito species are common throughout the hardwood and coniferous zones of Minnesota.

*Pigmental Encapsulation of* D. arbuta

Pigmental encapsulation of foreign bodies within the body of mosquitoes has been noted by many observers since Ross (1897) reported black spores in mosquitoes infected with malaria and Noe (1901) reported brown degeneration of *Dirofilaria immitis* in mosquitoes. Yen (1938), who also discussed the nature of these bodies, found encapsulated stages of *Dirofilaria immitis* chiefly in the malpighian tubules, blood content of the mid gut and in the hind gut of mosquitoes. The encapsulated forms of *Dipetalonema arbuta* in the present study were recovered chiefly from the body cavity and the fat body of the mosquito. Of the many observed none were seen which were intimately connected with the tracheal system of the mosquito. Most striking was the recovery of a live infective stage larva enclosed within the pigmented capsule of the second larval stage cuticle. This demonstrated that encapsulation of the living worm occurs. See Pl. II, Fig. 8.

Encapsulation of *D. arbuta* was encountered but rarely in *Aedes aegypti, A. canadensis, A. cinereus, A. excrucians,* and *A. fitchii.* In the last four of these species either microfilarial or first larval stages or both were occasionally found in the encapsulated condition. The encapsulated second larval stage was found in two specimens of *A. aegypti,* in one of *A. cinereus,* and in one of *A. fitchii.* In *A. vexans,* however, all the larval stages including the infective stage were found in the encapsulated condition, and encapsulation of the microfilarial, the first larval, and the second larval stages was common.

Normally the parasite matured to the infective stage within 9 to 11 days in 6 of our mosquito species under laboratory conditions in summer. In *A. vexans,* however, development was retarded, many of the parasites failed to develop, many died, and even after 60 days of incubation some had not developed beyond the first larval stage. The first and second larval stages were found long after the interval normally required for their transition into the next larval stage. The parasite failed to develop at the normal rate in *A. vexans,* and this failure was associated with a more frequent occurrence of encapsulated forms in all stages of development.

*Comparison of the Susceptibilities of Mosquitoes to* D. arbuta *Development*

Six local species of mosquitoes in Minnesota and one exotic species which were tested for their susceptibility to infection with *Dipetalonema arbuta* may be grouped as follows according to their fitness as intermediate hosts:

Class I. Efficient and susceptible vectors in which development is normal and larvae usually complete their development.

Group 1. Species in which no encapsulation was observed: *Aedes stimulans* and *Taeniorhynchus perturbans.*

Group 2. Species in which some larvae become encapsulated in the microfilarial (embryonic) stage or in the first larval stage: *Aedes canadensis.*

Group 3. Species in which some larvae become encapsulated in the microfilarial stage, in the first larval stage, or in the second larval stage: *Aedes aegypti, A. cinereus,* and *A. fitchii.*

Class II. Inefficient but occasionally susceptible vectors in which development is greatly retarded, larvae complete their development only rarely,

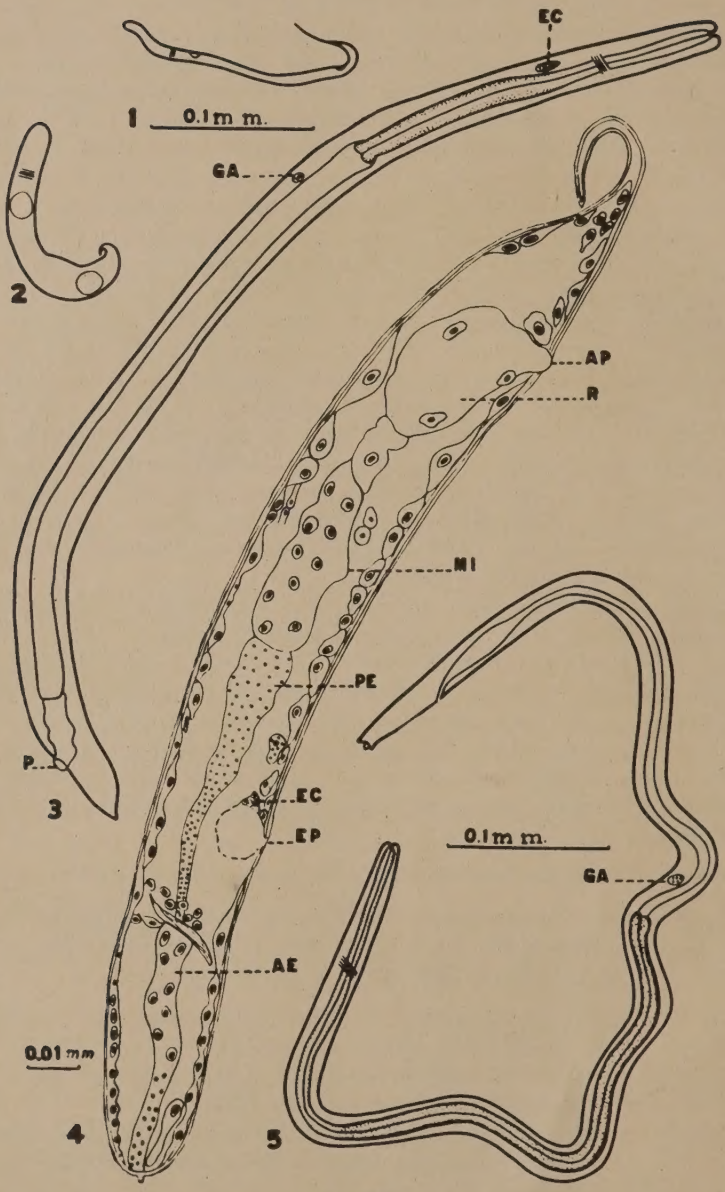


PLATE I

*Dipetalonema arbuta* Highby, 1943

FIG. 1. Microfilaria.
FIG. 2. 1st larval stage.
FIG. 3. 2nd larval stage.

encapsulation of the first 2 larval stages is common, and encapsulation of the third larval stage occurs: *Aedes vexans.*

Class III. Probable susceptible vector as indicated by normal development up to the third larval stage, third larval stage not demonstrated: *Aedes excrucians.*

### *Escape of the Infective Larvae from the Labium of the Mosquito*

Voluntary emergence of the infective stage of *D. arbuta* was induced by immersion of the labella in fresh warm porcupine serum without pressure, a method used by Blacklock (1926). Emergence was thus induced from all mosquito species tried, *A. aegypti, A. canadensis, A. cinereus,* and *A. fitchii.* In the first three species named the larvae emerged from the tip of the labella; from *A. fitchii* they emerged through the mesial surface between the tip and base of the labella. By this means as many as 12 worms were observed to make their escape from an infected mosquito in the course of a minute or two.

## INCIDENCE OF EXPERIMENTAL MOSQUITO INFECTIONS COMPARED TO FINAL HOST INFECTIONS

No correlation was noted between the rate of infection in the experimental mosquitoes and the degree of parasitism in the final host. One of the most lightly infected porcupines, infected with only one adult female *D. arbuta,* was the source of the greatest rate of mosquito infection, 81 per cent of 57 experimentally fed mosquitoes; while another equally light final host infection was the source of the least rate, 7 per cent of 27 experimental mosquitoes. The intermediate rates of 48 per cent (of 233 mosquitoes) and 70 per cent (of 20 mosquitoes) were from fairly heavy final host infections of 7 and 15 adult female worms respectively.

In this connection it will be noted that Hinman (1935) found no correlation between the number of microfilariae present at any one time in a sample of peripheral blood and the number of adult *Dirofilaria immitis* in the host.

## LARVAL DEVELOPMENT OF *Dipetalonema arbuta*

Within two hours after the mosquito had fed upon a porcupine infected with *D. arbuta* the microfilariae migrated into the abdominal cavity and fat body of the mosquito. Masses of them were seen to escape from the confines of the malpighian tubules at their distal tips. A few microfilariae were recovered from the head and thorax of the mosquito during the first 2 days of incubation, but most of them were found in the fat body and abdominal cavity.

*The first larval stage* was found most commonly in the fat body, frequently in the abdominal cavity, occasionally in the thorax and rarely in the head of the mosquito. It became almost quiescent on the 2nd day, a marked contrast to the sinuous lashing movement of the microfilaria. The former graceful action of the

FIG. 4. 1st larval stage from fresh specimen stained with Azur II.
FIG. 5. 3rd larval stage.
FIGS. 1, 2, 3 and 5 all drawn to same scale.

AE = anterior portion of esophagus
AP = anal pore
EC = excretory cell
EP = excretory pore
GA = Genitalanlage
MI = midintestine
P = anal plug
PE = posterior portion of esophagus
R = rectum

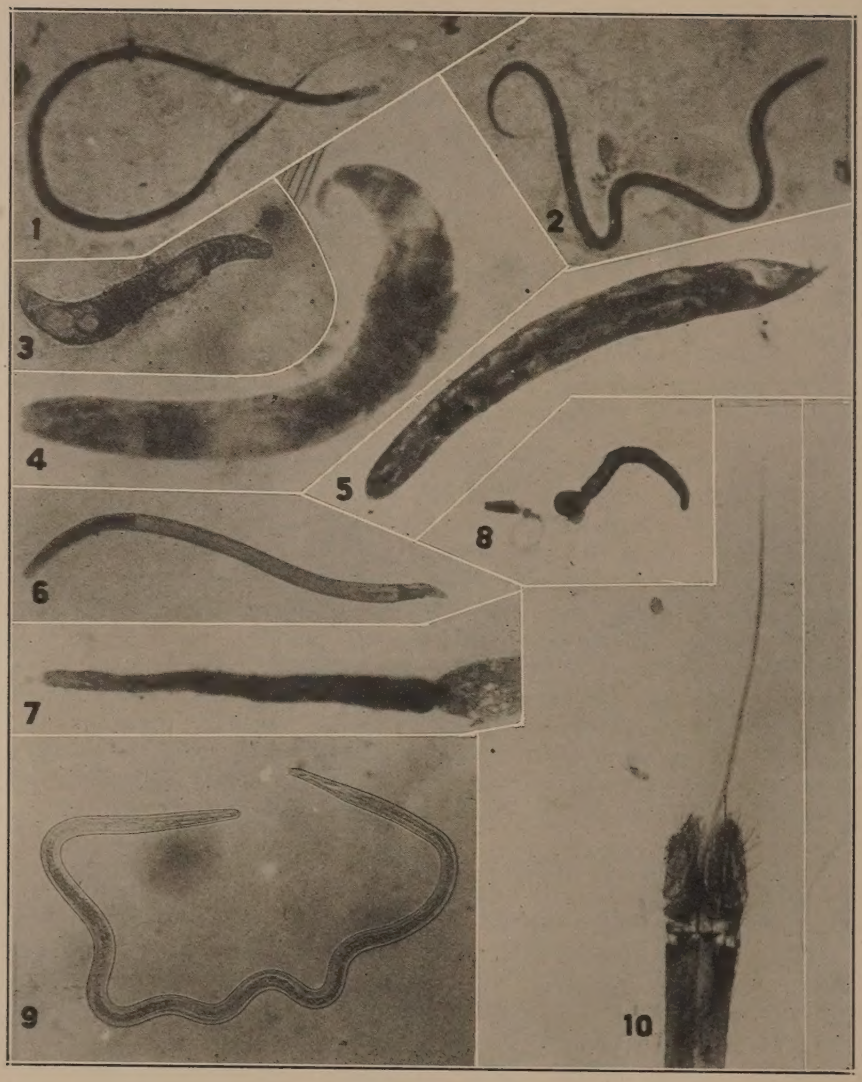


PLATE II

Developmental Stages of *Dipetalonema arbuta*

1. The microfilaria of *D. arbuta*, Wright's blood stain, about × 400.
2. The microfilaria of *Dirofilaria spinosa*, about × 400.
3. The 1st larval stage of *Dipetalonema arbuta* after 5 days in the mosquito, retarded development, beginning of encapsulation, about × 333.
4. The 1st larval stage, 2nd day, normal development, Ehrlich's hematoxylin, about × 675.
5. The 1st larval stage, after about 3 days,, normal development, fresh mount, Azur II, about × 333.
6. The 2nd larval stage still within 1st stage cuticle after 8 days normal development, Ehrlich's hematoxylin, about × 96.

worm was first modified by the appearance of a kink in its tail posterior to which the tail seemed to drag passively following the action of the body anterior to it.

The worm became shorter and wider so that by the 3rd day the smallest one noted was 90 μ long by 21 μ wide after fixation in hot Schaudin's fixative. It still retained the cuticle of the microfilaria and the slender posterior portion of the tail was crooked over. In its typical form it resembled a sausage and is called the "sausage stage" by some authors. Grossly it appeared to have two vacuoles and no well defined internal organization when observed in a fresh preparation (Pl. I, Fig. 2). After staining with Azur II, however, details of its anatomy could be seen (Pl. I, Fig. 4). The latter is shown in Pl. II, Fig. 5. Reference may be made to Plate II for photomicrographs of other larval stages.

By the 3rd and 4th days of incubation anlagen of the esophagus, intestine and rectum were clearly delineated, but no lumen had developed. No stoma was present. The esophagus was organized into two parts; its anterior portion was wider than the posterior portion at the junction of the two, but the posterior portion had a greater maximum width and was longer than the anterior portion. The esophagus was about twice as long as the intestine at this stage.

The anlage of the rectum appeared to consist of a syncytium of the 4 rectal cells about 14 μ long. Yamada (1927), Feng (1936), Kobayashi (1940), and Kotcher (1941) found in other filarioids that all 4 rectal cells developed into rectal structures. A structure similar to the anal plug described by Feng (1936) in *Microfilaria malayi* began to be evident in *D. arbuta* on the 3rd day as a rounded elevation at the site of the anal pore of the microfilarial stage. On the 4th day its rounded head was more protruding and proximally a hyaline tapered pointed spike curved anteriorly into the rectum anlage. On the 5th day the head of this anal plug stood out like a button. It persisted through the first and second larval stages.

The nerve ring was at the level of the junction between the two parts of the esophagus. The excretory pore opened into a vesicle of variable size on the posterior wall of which the excretory cell appeared with darkly staining nucleus. The cephalic space was almost obliterated by the third day. The muscle cells enlarged greatly and could be seen in the body wall of the worm.

After the initial shortening to 31 per cent of its original length and increase to 3.3 times its original width the first larval stage increased in size so that by the 5th day of incubation some specimens were 316 μ long by 22 μ wide, or about 1.1 times the length and 3.7 times the width of the mocrofilaria.

*The second larval stage* (Pl. I, Fig. 3) developed with or without shedding of the cuticle of the first larval stage. It grew in length and width and became from 553 to 742 μ long and 25 to 29 μ wide, or up to 2.5 times the length and 4.6 times the width of the microfilaria. The anterior extremity was bluntly rounded, and the tail short and conoid in marked contrast to the first larval stage.

The buccal orifice opened into a conoid stoma. The esophagus was 165 to 205 μ long. Its posterior portion had thicker walls and was about twice the length

7. The stoma, esophagus and part of the intestine of the 2nd larval stage, about × 300.

8. The 3rd larval stage within the broken pigmented capsule of the 2nd larval cuticle, about × 85.

9. The 3rd larval stage, fresh preparation, about × 120.

10. The 3rd larval stage fixed in the act of voluntary escape from the labium of the mosquito, after 10 days in the mosquito, about × 70.

of the narrower anterior portion. The lumen of the esophagus communicated with that of the intestine. The sides of the esophagus were slightly constricted where the intestine joined it; this resembled Feng's (1936) description for *Microfilaria malayi* more than that of Kotcher (1941) for *Foleyella* spp.

The intestine was thin-walled and wider than the esophagus. Its lumen frequently contained debris but did not communicate with the rectum. The rectum was about 36 μ long by 12 μ wide and was still plugged with the anal plug which could be dislodged from a fresh specimen by pressure on the cover glass. Its structure was continuous with that of the cuticle.

The Genitalanlage appeared as a small mass of cells about 7 μ in diameter in the body cavity about 43 μ posterior to the junction of the esophagus and intestine. The excretory cell was located about 30 μ posterior to the nerve ring at about the level of the junction of the anterior and posterior portions of the esophagus.

This stage was quiescent, almost motionless. It was usually recovered from the fat body of the mosquito. Occasionally specimens were found in the abdominal cavity, and rarely in the thorax and head of the mosquito. It commonly developed from the 5th to the 9th days of incubation.

*The third larval stage* (Pl. I, Fig. 5) appeared as early as the 9th day of incubation in the mosquito and was recovered alive as late as the 36th day. It was longer and more slender than the second larval stage and very active. Upon being released from the mosquito by dissection it thrashed about vigorously and rapidly crossed the field of the microscope. Within the mosquito's body it migrated freely from one part to another. It was seen to move leisurely down the mosquito's labium, probe at the tip of the labella, retreat back into the head, and a few minutes later it was recovered from the mosquito's abdomen. These larvae were usually recovered from the labium, head, thorax and abdomen of mosquitoes, and rarely from the palps and legs. They quickly escaped voluntarily from the tip of the labella when it was immersed in fresh warm porcupine serum. See Pl. II, Fig. 10.

The third larval stage was 0.88 to 1.16 mm long and 16 to 18 μ wide, 3 to 4 times the length and 2.5 to 3 times the width of the microfilaria. The cuticle was much thicker than that of the earlier larval stages. Its body width was almost uniform except for slight attenuation anterior to the nerve ring and posterior to the anus. The anterior extremity was bluntly rounded and the posterior extremity had 3 papilla-like structures.

The mouth opened at the anterior extremity. The stoma was about 7 μ long with highly refractive walls. The esophagus was about 553 μ long, 58 per cent of the worm's length, thus differing from that of *M. malayi* which was about 36 per cent according to Feng (1936). The narrower anterior portion of the esophagus was about 20 per cent of the worm's length. The wider posterior portion had cells which stained more deeply with haematoxylin.

The intestine was narrower than the posterior portion of the esophagus. The lumina of the rectum and intestine communicated. The rectum in its mid-region was wider than the intestine, but tapered to the anus, which was about 64 μ from the posterior extremity. The anal plug of the first and second stage larvae was not present.

The Genitalanlage appeared as a small mass of cells between the intestine and the ventral body wall at a point about 30 μ posterior to the junction of the esopha-

gus and the intestine. The location of the excretory pore was noted after vital staining of a fresh specimen with methylene blue. Its position was about 50 μ posterior to the nerve ring. The nerve ring was 72 to 86 μ from the anterior end, about 7.5 per cent of the worm's length.

The larval development of *D. arbuta* is fundamentally similar to that described for other filarioid worms. The site of development differs as this is the first of the filarioids reported to develop in the fat body of the mosquito. *Wuchereria bancrofti, W. malayi,* and *Foleyella* develop in the thoracic muscles of the mosquito according to Manson (1884). Feng (1936) and Kotcher (1941). *Dirofilaria immitis* and *D. repens* develop in the malpighian tubules of the mosquito according to Grassi and Noe (1900) and Fülleborn (1908). In flies of the genus *Chrysops,* however, development of *Loa loa* in the fat body was reported by Kleine (1915) and Connal and Connal (1922). An interesting comparison of developmental sites is drawn by Chandler et al (1940) with the spiruroid worms, *Habronema* which develop in the fat body and *Draschia* in the malpighian tubules of muscoid fly maggots.

Two moults occur in the development of *D. arbuta* as reported for other filarioids by Yamada (1927). Buckley (1934). Feng (1936), Kobayashi (1940) and Kotcher (1941).

## SUMMARY

1. Porcupines from northern Minnesota were found commonly infected with two species of filarioid nematodes, *Dipetalonema arbuta* and *Dirofilaria spinosa.*

2. The microfilariae of these two species could be distinguished from each other by three anatomical characters.

3. The natural incidence of filariasis in selected species of wild mosquitoes from the vicinity of Minneapolis and St. Paul, Minnesota, was none in 340 specimens examined.

4. Six species of Minnesota mosquitoes and one exotic species were susceptible to the complete larval development of *Dipetalonema arbuta.*

5. Marked resistance to the development of the parasite correlated with its pigmented encapsulation was noted in *Aedes vexans.*

6. Pigmented encapsulation was noted to have occurred on a living worm.

7. Voluntary emergence of the infective larvae from the mosquito's labium was observed in 4 mosquito species.

8. No correlation was found between the rate of infection in experimental mosquitoes and the degree of parasitism in the final host source of infection.

9. The site of development was in the fat body, hitherto unreported for filarioids in mosquitoes.

10. The larval development of *D. arbuta* is fundamentally similar to that described for other filarioids.

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# VECTORS, TRANSMISSION, DEVELOPMENT, AND INCIDENCE OF *DIROFILARIA SCAPICEPS* (LEIDY, 1886) (NEMATODA) FROM THE SNOWSHOE HARE IN MINNESOTA

Paul R. Highby

Microfilariae were recovered from the blood contained in the gut of an engorged tick removed from the ear of a snowshoe hare, *Lepus americanus phaeonotus* Allen, which was captured near Baudette, Minnesota. The hare, from which subsequently 8 adult female and 7 adult male *Dirofilaria scapiceps* (Leidy, 1886) were recovered, was subjected to feeding experiments with mosquitoes in order to determine some of the susceptible vectors of the parasite, and to transmit the infection experimentally.

## EXPERIMENTALLY SUSCEPTIBLE MOSQUITOES

Captured wild mosquitoes were used in these experiments. Larval filariasis was found in 15 per cent of 139 mosquitoes which were examined by dissection after feeding on the infected hare, and in 15 per cent of 54 mosquitoes examined alive by light transmitted through the labium. That these mosquitoes became infected by feeding on the hare may be inferred by comparison with the natural incidence rate of no filariasis in 340 local wild mosquitoes taken in the same locality (Highby, 1943).

Only those experimentally fed mosquitoes from which the 3rd larval stage of the parasite was recovered by dissection or seen moving within the labium were considered to be susceptible to the complete development of *D. scapiceps.*

In *Aedes canadensis* Theobald the 3rd larval stage was first found on the 12th day of incubation. Of 63 mosquitoes examined after the 11th day of incubation 10 were found to have the 3rd larval stage. Of these, 50 were examined by dissection and 12 by transmitted light through the labium.

In *A. excrucians* Walker the 3rd larval stage was found as early as the 11th day of incubation. It was found in 3 out of 6 mosquitoes which survived the minimum incubation period. Five were examined by dissection and 1 by examination of the labium.

In *A. fitchii* Felt and Young development was completed on the 12th day of incubation. The 3rd larval stage was found in 5 out of 16 mosquitoes examined after the 11th day of incubation. Thirteen were examined by dissection and 3 by examination of the labium.

In *A. vexans* Meigen the 3rd larval stage was found on the 11th day of incubation. Forty-four mosquitoes survived the 11-day incubation period. The 3rd larval stage was found in 1 out of 16 dissected mosquitoes. None were seen by examination of the labium of the remaining 28.

Some evidence of resistance to the development of *D. scapiceps* in *A. canadensis* was noted in the occurrence of pigmental encapsulation of the 1st larval stage and in the occurrence of retarded development as shown by the finding of the 1st larval stage as late as the 14th day and the 2nd larval stage as late as the 13th day of incubation.

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A comparison of the relative susceptibilities of the different species of mosquitoes in this series of experiments is difficult because of the lightness of the infections in the mosquitoes. The heaviest infection encountered in the series of 139 dissections was of 5 infective stage larvae taken from *A. excrucians* on the 11th day.

Mosquito infections were diagnosed as frequently by examination of the labium (15 per cent) as by dissection (15 per cent).

In addition to the 4 species mentioned above which were proved to be susceptible vectors of *D. scapiceps* a 5th species, *A. cinereus* Meigen, was demonstrated to be a transmitter on the basis of a transmission experiment described below.

## EXPERIMENTAL TRANSMISSION OF *D. scapiceps* INFECTION TO DOMESTIC RABBITS

Two methods were employed in experiments to infect domestic rabbits with the 3rd larval stage of *D. scapiceps* from the infected mosquitoes; one method was to allow experimentally infected mosquitoes to bite the rabbit and the other was to transfer the larval worm mechanically from the mosquito to the rabbit.

### *Experimental Transmission by Mosquito Bites*

Six domestic rabbits were subjected to infection from the bites of mosquitoes which had fed upon the infected snowshoe hare at least 13 days previously. Blood samples from the experimental rabbits were then examined periodically for the appearance of microfilariae. From 2 of the 6 experimental rabbits microfilariae were recovered.

### *Experiment with* Aedes fitchii *Felt and Young*

A domestic rabbit was subjected to the bites of 5 specimens of *A. fitchii*, all of which had fed on the infected snowshoe hare not less than 12 days nor more than 17 days previously. (The untimely death of the hare precluded procurement of a greater number of experimentally infected mosquitoes.) These mosquitoes had been examined and in 2 of them the infective stage larvae had been seen moving in the labium. All 5 mosquitoes fed on the experimental rabbit July 18, 1938, and after a 3 day interval fed again, after which they were dissected. From one mosquito 3 infective stage larvae were recovered but the other 4 had no worms. At least one mosquito had been relieved of its infective larvae since 2 were known to have been infected and only one retained infective larvae after the experimental feeding on the prospective final host.

During the ensuing year the peripheral blood of this rabbit was examined periodically for the presence of microfilariae in fresh smear preparations and in centrifuged hemolysed preparations.

A microfilaria was found in a fresh preparation of blood drawn from this rabbit's ear 286 days after it was subjected to infection by the bites of the infected mosquitoes. Thirty days later xenodiagnosis of this rabbit with mosquitoes resulted in the recovery of a microfilaria from the blood content of the mosquito's mid gut. Post mortem examination of the rabbit on the 353rd day yielded no adult worms but a microfilaria was recovered from its lung.

Recovery of 3 microfilariae from this rabbit constitutes evidence of the transmission of *D. scapiceps* from the snowshoe hare to the domestic rabbit by the bites of experimentally infected *A. fitchii*.

### *Experiment with* Aedes cinereus *Meigen*

Another domestic rabbit was subjected to experimental transmission of *D. scapiceps* infection by the bites of 3 specimens of *A. cinereus* July 19, 1938. These mosquitoes had fed not less than 13 days nor more than 18 days previously upon the infected snowshoe hare. In the course of subsequent periodic examinations a microfilaria was recovered from the peripheral blood of this rabbit 391 days after the experimental exposure to the infection. Upon post mortem examination 30 days later no adult *D. scapiceps* were recovered. The recovery of the microfilaria from the rabbit's blood, however, constitutes evidence of the experimental transmission of *D. scapiceps* from the snowshoe hare to the domestic rabbit by the bites of *A. cinereus*.

### *Experimental Mechanical Transmission*

A domestic rabbit was infected mechanically with 6 infective stage larvae recovered by dissection from 2 mosquitoes which had fed from 11 to 13 days previously on the infected snowshoe hare. These larvae were placed into an abrasion in the skin of the rabbit July 19, 1938. The rabbit was operated upon 240 days later and from its tarsal bursa one adult male *D. scapiceps* was recovered.

### *Control Animals*

A domestic rabbit selected for control died 6 months after initiation of the experiment. Examination for developmental stages and adult worms yielded no specimens.

Four other domestic rabbits which were subjected to experimental transmission by the bites of 4 species of experimentally engorged mosquitoes, were examined for microfilariae and adult worms in the same way as the others but with negative results. The fact that no evidence of transmission materialized in these rabbits would not operate to exclude the mosquito species tried as potential vectors because of the small number of mosquitoes used. These 4 rabbits may, however, be considered as successful controls.

### *Conclusions Drawn from Transmission Experiments*

As a result of these experiments it was demonstrated that the microfilaria of *D. scapiceps* from the infected snowshoe hare can develop in the mosquito to the infective stage, be transferred mechanically to the scarified skin of a domestic rabbit and in the latter host develop to the adult form in 240 days.

It was further demonstrated that *D. scapiceps* infection of the snowshoe hare can be transmitted to domestic rabbits by the bites of *Aedes excrucians* and *A. cinereus* as evidenced by the recovery of microfilariae from the blood of the second definitive host 286 to 391 days after exposure to the infection.

In connection with this experiment 5 control rabbits remained free of the infection.

### *Discussion of Transmission Experiments*

Hitherto only one case of successful controlled experimental transmission of filarioids to new hosts by the bite of infected mosquitoes has been demonstrated. Bancroft (1904) transmitted *Dirofilaria immitis* by infected mosquitoes and recovered adult worms after 7 months from one dog and both microfilaria and adult worms after 9 months from another. Two control dogs remained uninfected for 2 years.

The report of Grassi and Noe (1900) of the recovery of the anterior half of an immature adult *D. immitis* 16 days after mechanical inoculation of a dog with infective larvae from a mosquito was severely criticized by Annett, Dutton and Elliott (1901). Noe's (1900) uncontrolled effort at transmission of *D. immitis* by mosquito bite appeared to be very unsatisfactory to Sambon (1902). The dog was not selected and the mosquitoes were not subjected to controlled feeding upon a source of infection.

Fülleborn (1929) reported transmission of *D. immitis* by mechanical inoculation with recovery of microfilariae after 8 months from 4 out of 5 dogs and adult female worms from the 5th dog.

Failure attended the efforts of Hinman (1935) to transmit *D. immitis* through *Aedes aegypti*. Connal and Connal (1922) failed to transmit *Loa loa* to laboratory animals. No further report has come from Poynton and Hodgkin (1939) regarding their attempt to obtain the adult of *M. malayi* by transmission from man to monkey.

Yokogawa (1939) reported an infection experiment with *Wuchereria bancrofti* on 5 volunteers. In one case 82 days after infection, and in another 111 days after the first and 56 days after a second infection, 2 microfilariae were recovered from 2.0 ml of blood examined at night. He believed that larvae may become adult and produce microfilariae 20 to 30 days after infection. It would seem that an account should have been given of precautions taken to avoid infection of the volunteers previous to the experiment.

## NOTES ON THE LARVAL DEVELOPMENT OF *D. scapiceps*

The developmental stages of *D. scapiceps* in the mosquito are much similar to those of *Dipetalonema arbuta* and of other filarioids which have been reported. Within 9 hours after ingestion the microfilariae were found exsheathed in the body cavity. Escape from the microfilarial sheath may occur in the body cavity. One was recovered from the body cavity half-way free of its sheath and entangled with the mosquito's tracheal system during the 1st day of incubation. In this respect the development of *D. scapiceps* would differ from that of *Wuchereria bancrofti* which escapes from its sheath in the mid gut according to Manson (1884), Yamada (1927), and Kobayashi (1940).

The 1st and 2nd larval stages were recovered from the fat body of the mosquito.

The 3rd larval stage was 780 μ long by 14 μ wide. Three blunt papillae were present at its posterior extremity. Its earliest appearance was on the 11th day of incubation in the mosquito. It was recovered from the labium, head, thorax, body cavity and fat body.

## THE MICROFILARIA OF *D. scapiceps*

Microfilariae in rabbits from North America have been reported 3 times previously. The occurrence of microfilariae in the blood of a rabbit from British Columbia was reported by Harkin (1927). Schwartz and Alicata (1931) described microfilariae from the blood of *Lepus washingtonii* from the state of Washington, and conjectured the probability of their being the larvae of *D. scapiceps*. In *L. americanus* from Ontario microfilariae were reported by MacLulich (1937).

In connection with this investigation I studied specimens of microfilariae obtained from the lymph at the site of infection, the tarsal bursa of a snowshoe hare

infected with 8 female and 7 male adult *D. scapiceps*. The specimens were not allowed to dry but were studied in fresh preparations of aqueous Azur II.

Microfilaria of *Dirofilaria scapiceps*
(Fig. 1)

Microfilaria sheathed, finely striated transversely. Length 205 to 270 μ (243 μ).* Width 6 μ. Anterior end with protrusible point, bluntly rounded when retracted. Tail attenuated with blunt tip. Cephalic space 3 μ long. First 4 nuclei of nuclear column present scattered appearance. Position of anatomical points in terms of distance from anterior extremity expressed in percentage

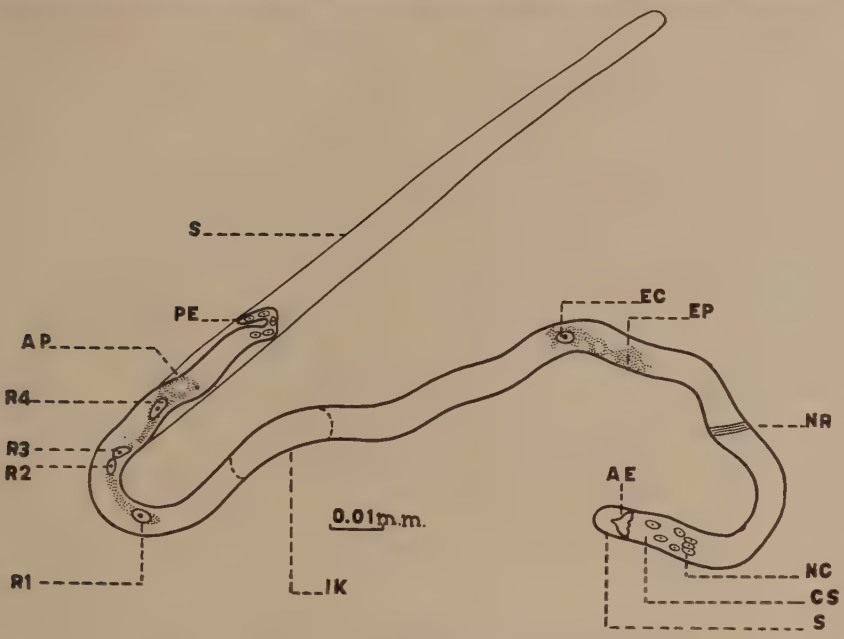


FIG. 1. Microfilaria of *Dirofilaria scapiceps* (Leidy, 1886).

AE—anterior extremity
AP—anal pore
EC—excretory cell
EP—excretory pore
CS—cephalic space
IK—"Innen Körper" location
NC—anterior elements of nuclear column (only a few of the most anterior and most posterior nuclei are shown).
NR—nerve ring
PE—posterior extremity
$R_1$, $R_2$, $R_3$, $R_4$—1st, 2nd, 3rd, and 4th rectal cells
S—sheath

of the worm's entire length: nerve ring 20.8 to 24.5 (22.7); excretory pore 31.2 to 36.6 (33.2); excretory cell 36.8 to 42.7 (39.2); "Innen Körper" anterior limit 51.6 to 61.2 (55.2), posterior limit 56.9 to 68.8 (63.4), length 3.1 to 17.1 (8.9); 1st rectal cell 68.7 to 75.6 (72.2); and anal pore 82.0 to 86.0 (84.1). Last 4 to 7 nuclei at posterior end of nuclear column arranged in linear series, and become progressively more attenuated toward posterior end. Last 3 nuclei scattered with clear spaces between them. Last nucleus more rounded, located at extreme tip of tail and gives tail tip blunt appearance.

A comparison of the description given above with that given for *Mf.* species (? *Dirofilaria scapiceps*) by Schwartz and Alicata (1931) reveals certain differences

*Figures in parentheses denote average of 20 specimens measured in aqueous Azur II; except "Innen Körper," average of 9 specimens.

as well as similarities. Perhaps the differences might appear less pronounced if material from the 2 sources, *L. americanus phaeonotus* and *L. washingtonii*, were prepared for study by similar treatment. The writer cannot determine these larvae as being specifically identical from objective consideration of the material at hand.

## INCIDENCE AND DISTRIBUTION OF *D. scapiceps* INFECTION IN SNOWSHOE HARES IN MINNESOTA

Snowshoe hares from 3 widely separated sections representing the eastern, northwestern, and southwestern extremes of the range of the hare in Minnesota were examined in 1938–1939. From the Lake Superior watershed in Lake County 18 infections were found in 31 hares (58 per cent). From Lake of the Woods County 2 infections were found in 15 hares (13 per cent). From this region Manweiler (1938) reported infection of "approximately one-third of all specimens of snowshoe hares examined" in the winters of 1936–37 and 1937–38. From Morrison County in central Minnesota 3 infections were found in 11 hares (27 per cent). This sampling indicates that the infection was common throughout the range of the snowshoe hare in Minnesota in 1938–39.

A total of 57 hares were examined and 23 cases of infection were found. From these infections 176 adult *D. scapiceps* were recovered of which 106 were females and 70 males. In addition there were 8 cases of calcification of the worms all of which developed in hares from the Lake County region. No worms were found in the fore feet, subcutaneously in the lumbar region, or in the intermuscular fascia of the large muscles of the leg, shoulder, and back. Except for one case the parasites were all found in the tarsal bursa, a pocket below the distal end of the tibia on the anterior aspect of the tarsal joint covered with tough connective tissue. The exception noted was one recovered from under connective tissue on the posterior aspect of the tarsal joint. The heaviest infection was 18 worms, 9 males and 9 females in one case, and 6 males and 12 females in another.

## SUMMARY

1. Five species of Minnesota mosquitoes were demonstrated to be susceptible to the complete larval development of *Dirofilaria scapiceps*. The susceptible species are: *Aedes canadensis, A. cinereus, A. excrucians, A. fitchii* and *A. vexans*.

2. Transmission of *D. scapiceps* from the snowshoe hare to the domestic rabbit was accomplished by mechanical transfer of the infective stage larvae from experimentally infected mosquitoes to the scarified skin of the rabbit, and resulted in the recovery of an adult *D. scapiceps* from the experimental rabbit 240 days later.

3. Transmission of *D. scapiceps* from the snowshoe hare to domestic rabbits by the bite of experimentally infected mosquitoes was accomplished through *A. fitchii* and *A. cinereus*.

4. Microfilariae were demonstrated in the blood of 2 experimentally infected domestic rabbits 286 to 391 days after exposure to infection.

5. The transmission experiments were controlled by 5 rabbits which remained free of the infection.

6. Larval filariasis in mosquitoes may be detected as frequently by examination of the labium of the living mosquito as by the method of dissection.

7. The larval stages of *D. scapiceps* were grossly similar to those of other filarioids.

8. The microfilaria may escape from its sheath while in the body cavity of the mosquito.

9. The 1st and 2nd larval stages were found in the fat body of the mosquito.

10. The earliest noted appearance of the 3rd larval stage was on the 11th day of incubation in the mosquito.

11. The microfilaria of *D. scapiceps* is described and figured.

12. *D. scapiceps* infection of the snowshoe hare is common throughout the range of the hare in Minnesota as shown by incidence rates of 13 to 58 per cent in samples from 3 geographic extremes of its range.

13. Xenodiagnosis of filariasis by ticks and mosquitoes was useful.

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# BLOOD PROTOZOA OF BIRDS TRAPPED AT ATHENS, GEORGIA*

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A considerable amount of information concerning the incidence of blood parasites in wild birds in certain sections of the United States has been presented by various investigators. Among these Huff (1939) reported on smears from 967 birds from widely different areas, Manwell and Herman (1935) examined 652 birds at Syracuse, New York, and Cape Cod, Massachusetts, and Wetmore (1941) 618 birds in the District of Columbia and Patuxent Refuge vicinity. Less extensive surveys were carried out by Coatney and Jellison (1940) on 20 birds taken in Beaverhead County, Montana, by Coatney and West (1938) on 84 birds in Nebraska, by Herms et al. on 150 birds in California, and by Paul E. Thompson on 275 birds trapped in Bulloch and Clarke Counties, Georgia (1943).

The author, during the last two years, has examined blood smears from 1103 birds trapped and banded on the campus of the University of Georgia at Athens. Smears from these birds were stained with MacNeal's tetrachrome and examined with 15X oculars and 1.8 mm oil immersion objective for at least ten minutes in each case.

Table 1 gives the incidence of *Haemoproteus, Plasmodium* sp. and *Trypanosoma* found.

TABLE 1.—*Distribution of blood protozoa in birds examined at Athens, Georgia*

| Host | | Parasite | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Scientific name | Common name | Negative | *Plasmodium* sp. | *P. relictum* | *P. cathemerium* | *P. elongatum* | *P. hexamerium* | *P. vaughani* | Total *Plasmodium* | *Haemoproteus* | *Trypanosoma* | Total birds |
| *Passer domestica* | English sparrow | 418 | 5 | 42 | 14 | 9 | .. | .. | 70 | .. | 1 | 489 |
| *Toxostoma rufum* | Brown thrasher | 111 | 11 | 2 | 4 | 22 | 2 | .. | 41 | 63 | .. | 215* |
| *Cyanocitta cristata* | Blue jay | 56 | .. | .. | .. | .. | .. | .. | .. | 24 | .. | 80 |
| *Zonotrichia albicollis* | White throated sparrow | 78 | .. | .. | .. | .. | .. | .. | .. | 32 | 1 | 110* |
| *Mimus polyglottos* | Mocking bird | 29 | 3 | 2 | .. | 1 | .. | .. | 6 | 31 | .. | 66 |
| *Pipilo erythrophthalmus* | Towhee | 13 | 8 | 2 | .. | .. | 5 | .. | 15 | 12 | 1 | 40* |
| *Richmondena cardinalis* | Cardinal | 7 | 4 | 8 | 1 | 5 | .. | .. | 18 | 7 | 2 | 25* |
| *Sturnus vulgaris* | Starling | 16 | .. | .. | .. | .. | .. | .. | .. | .. | .. | 16 |
| *Quiscalus quiscula* | Purple grackle | 1 | .. | 1 | .. | .. | 1 | .. | 2 | 1 | .. | 3* |
| *Spizella passerina* | Chipping sparrow | 22 | .. | .. | .. | .. | .. | .. | .. | 11 | .. | 33 |
| *Turdus migratorius* | Robin | 2 | .. | .. | 1 | .. | 1 | 1 | 3 | .. | .. | 4* |
| *Dumetella carolinensis* | Catbird | 5 | 1 | 1 | .. | .. | .. | .. | 2 | .. | .. | 7 |
| *Spizella pusilla* | Field sparrow | 1 | 1 | 1 | .. | 1 | .. | .. | 3 | .. | .. | 3* |
| Total | | 759 | .. | .. | .. | .. | .. | .. | .. | .. | .. | 1091 |

* Double infections.

In addition to birds listed in Table 1, two humming birds, one red-headed woodpecker, one loggerhead shrike, one redstart, one prairie warbler, one hairy woodpecker, one domestic pigeon, and one gray-cheeked thrush were all negative; one

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flicker, and one blue bird showed an infection with *Haemoproteus,* and one wood-thrush *P. cathemerium.*

These studies appear to include 13 new host records for *Plasmodium* and 1 for *Haemoproteus* not present in the check-lists of Coatney (1936), Coatney and Roudabush (1936), and the above mentioned surveys, as follows: *Plasmodium elongatum* Huff, *P. hexamerium* Huff, *P. relictum* Grassi and Feletti, and *P. cathemerium* Hartman, in the brown thrasher, *Toxostoma rufum; P. relictum* and *P. elongatum* in the mocking bird, *Mimus polyglottos; P. relictum, P. hexamerium,* and *Haemoproteus* in the towhee, *Pipilo erythrophthalmus; P. cathemerium* in the cardinal, *Richmondena cardinalis;* and *P. cathmerium* and *P. hexamerium* in the robin, *Turdus migratorius; P. relictum* in the catbird, *Dumetella carolinensis; P. hexamerium* in the purple grackle, *Quiscalus quiscula;* and *P. elongatum* and *P. relictum* in the field sparrow, *Spizella pusilla.*

In most instances the number of birds of a single species taken within a limited period was too small to make possible an adequate picture of the incidence of parasites for that particular host by seasons. In the case of *Plasmodium* in English sparrows, *Haemoproteus* in jays, and *Plasmodium* and *Haemoproteus* in brown thrashers, however, enough individuals were taken to make a few comparisons. These observations are recorded in Table 2 and graphically shown in Fig. 1.

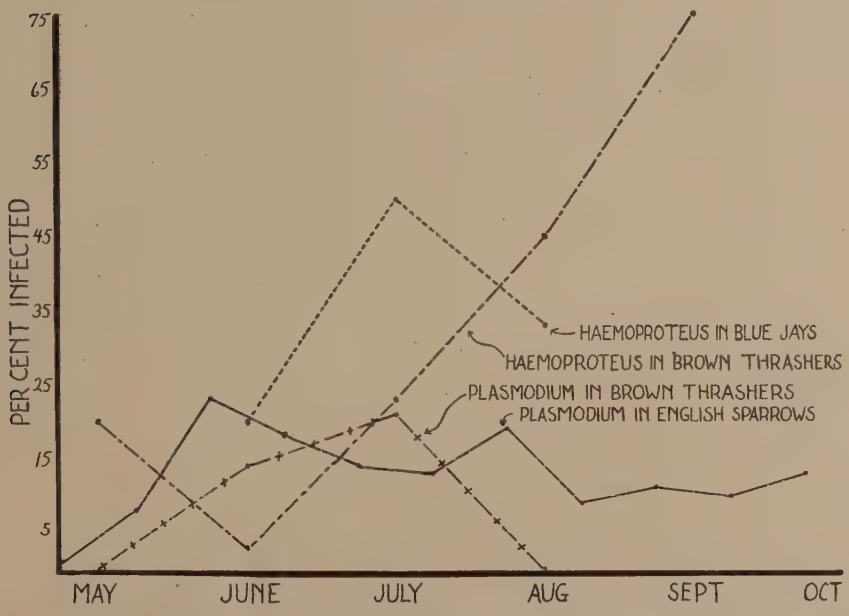


FIG. 1. Graph showing the incidence of *Plasmodium* and *Haemoproteus* in blue jays and brown thrashers from May through September, and of *Plasmodium* in the English sparrow from May through October.

These figures show that there are rather abrupt changes in incidence from month to month which make comparisons of incidence in different regions meaningless unless one also has information on the dates of examination.

Some of the infections were diagnosed on the basis of examination of one slide only, and some of the infections were diagnosed on the basis of examinations of several slides made at different times of day over a period of two or three days. Subinoculation into canaries was carried out in the following instances: *P. hexamerium* from the towhee, *P. relictum* from the cardinal, towhee, and purple grackle; *P. cathemerium* from the robin and towhee. One of the *P. relictum* infections in an English sparrow was observed at intervals for three days and found to show the matinal schizogony described by Huff (1937) in the case of a strain of *P. relictum* isolated from a robin.

TABLE 2

| Host and parasite | May | | June* | | July | | August | |
|---|---|---|---|---|---|---|---|---|
| | No. | % | No. | % | No. | % | No. | % |
| English sparrow *Plasmodium* .... | 71 | 1 | 44 | 19 | 93 | 16 | 113 | 16 |
| Brown thrasher *Plasmodium* .... | 5 | 0 | 36 | 14 | 38 | 21 | 27 | 0 |
| Brown thrasher *Haemoproteus* .. | 5 | 20 | 36 | 3 | 38 | 23 | 27 | 45 |
| Blue jay *Haemoproteus* ..... | † | .. | 10 | 20 | 28 | 50 | 15 | 33 |

* During the first two weeks of June the infection reached a peak of 23%.
† No jays examined in May, but 16 caught in February, March, and April were all negative.

*Plasmodium relictum* seems to be not only the most widely distributed species of the avian malaria parasite but also the most abundant. Of all the plasmodia found in birds examined in Athens from March through August, 1940, and from March through October, 1941, 37% were *P. relictum*; 24% *P. elongatum*; 13% *P. cathemerium*; and 6% *P. hexamerium*; 21% were of undetermined species. Only one infection of *P. vaughani* was found. This was in a young robin.

Table 1 shows that of the 12 recognized species of avian malaria parasites *P. relictum*, *P. elongatum*, *P. cathemerium*, *P. hexamerium*, and *P. vaughani* were found in birds at Athens, Georgia. Other investigators have reported *P. nucleophilum*, *P. circumflexum*, *P. polare*, and *P. oti* from the United States. That our survey includes none of these plasmodia may be a result of our inclusion of very few or none of the host from which these species were recorded.

About 1 out of 7 birds showed infection with plasmodia during the season covered in our survey. Many of them showed double infections. Two triple infections in the case of young female cardinals were noted. Double infections were encountered as follows:

English sparrow ..... *P. relictum* and *P. elongatum*; *P. relictum* and *P. cathemerium*.
Brown thrasher ..... *P. elongatum* and *Haemoproteus*.
White throated sparrow ..... *Haemoproteus* and *Trypanosoma*.
Mocking bird ..... *Haemoproteus* and *P. relictum*.
Towhee ..... *Haemoproteus* and *Trypanosoma*; *P. hexamerium* and *P. relictum*.
Cardinal ..... *P. cathemerium* and *Haemoproteus*; *P. cathemerium*, *P. relictum*, and *P. elongatum*; *Trypanosoma*, *P. relictum*, and *Haemoproteus*; *P. relictum* and *P.*

*elongatum; P. relictum* and *Haemoproteus;* and *P. relictum* and *Trypanosoma.*

Purple grackle ..... *Haemoproteus, P. hexamerium,* and *P. relictum.*


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# OVERWINTER LOSS OF NODULAR WORM LARVAE FROM A SHEEP PASTURE AND ITS BEARING ON THE CONTROL OF NODULAR WORM DISEASE[1]


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In the development of methods for the control and eradication of ruminant parasites the importance of directing attention to the free-living stages which occur on pastures as well as to the parasitic stages which develop within the host has been repeatedly emphasized. One of the methods advocated for ridding pastures of the free-living stages of parasitic worms has been the "resting" of pastures for a number of months or over winter to allow the free-living stages to die off. Existing information as to the value of this method in the control of particular species of parasites is insufficient to formulate a sound control program for sheep parasites as a whole. The present report concerns an experiment devised to test the value of resting pastures overwinter as a measure for controlling nodular worms in sheep.

## REVIEW OF WORK OF PREVIOUS INVESTIGATORS

In Louisiana, Dalrymple (1904) observed that a pasture on which 2 lambs became infected in August did not contain infective larvae the following June, since 3 lambs which grazed on it at that time failed to become infected. In this case the survival test was complicated by the fact that the pasture had been plowed, seeded, and a crop harvested during the interval.

Ransom (1906) noted the failure of nodular worm larvae to survive overwinter from October to June on pastures on high ground and on low, swampy ground in Maryland.

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[1] The experiment reported in this paper was carried out at the United States Department of Agriculture, Beltsville Research Center, Beltsville, Md.

Smith and Ring (1927) found that a clean flock of sheep did not acquire nodular worm infection when put on a previously infested swampy pasture in New Jersey which had been rested from November of one year to May of the second year.

Rebrassier (1933) gave an abstract of an experiment in which nodular worm larvae survived at least one winter on a pasture in Ohio, as determined by placing susceptible lambs on it.

Baker (1938, 1939) listed this parasite among the sheep nematodes which survived overwinter from July to the following June on a hillside pasture in New York. It is impossible to tell from his published data whether or not the nodular worm was one of the "strongyles" which he reported in a second experiment to have survived "at least 21 months."

In Canada, Swales (1940) found that nodular worm larvae died out overwinter on pastures, as shown by failure of spring lambs to become infected in June on pastures known to have been contaminated the previous autumn.

Shorb (1942) found that nodular worm larvae failed to survive on experimental pasture plots at Beltsville, Maryland, for more than $2\frac{1}{2}$ months in summer, or $3\frac{1}{2}$ months in summer and fall, and that the larvae of this parasite did not apparently develop on pastures in the late fall, winter and spring.

## MATERIALS AND METHODS

1. *The experimental pasture.*—The pasture used was 0.7 acre in size and was separated on two sides from the adjacent, unoccupied paddocks by a high crowned road and on the other two sides by a ditch between a double fence. The pasture was shaded in spots by 46 scattered trees—mostly young chestnut-oak and red oak. The pasture sloped gently toward the northeast. Situations regarded as especially favorable for the development and survival of nematode larvae existed in the shade on two sides of the pasture; in the masses of fecal pellets which accumulated during the contamination period at the edge of bare areas under the trees that were frequented by the sheep; and in the shade of the A-type shelter where feces accumulated at first in the grass and later on the bare, packed ground. The soil was of a poor quality, heavy sandy loam to almost a clay loam type. The forage was relatively sparse and consisted of mixed pasture grasses, clovers, and weeds. Special precautions were taken to guard against accidental contamination, from any extraneous source, of the pasture, feed, and water. Only two persons entered the pasture, and only after putting on carefully scrubbed and rinsed rubbers.

2. *Experimental animals.*—All the sheep used in the experiment had been raised in stalls until weaning time, when they were put into raised, concrete-floored cages outdoors. Both stalls and cages were carefully cleaned and allowed to dry thoroughly before the lambs were placed in them. Bedding was removed with the accumulated feces three times each week. Although the ewes from which the experimental lambs were raised were not free from worms, this method of raising lambs, with only rare exceptions, proved at this station to be satisfactory for the prevention of all parasitic infection, except the almost ubiquitous coccidia and *Strongyloides*. Special precautions were taken to prevent accidental infection of the 6 test lambs. Chance of infection passing from ewes to lambs before weaning time was minimized because the ewes had been treated with phenothiazine the previous fall and again just before parturition, and also because the lambs were weaned when they were 7 to 8 weeks

old. The cage into which the lambs were put had been carefully scrubbed with chloride of lime.

3. *Evidence of freedom of the test lambs from accidental infection.*—The first fecal examination, made on the 6 test lambs at the time they were weaned, revealed numerous coccidial oöcysts and small numbers of *Strongyloides* eggs. A second examination 26 days later gave similar results, cultures of 1 to 2 grams of feces from each lamb yielding only small numbers of *Strongyloides* larvae. Even though no other species of worms were found, it was decided a week later to reduce still further the chance of an accidental infection by treating each of the 6 lambs with 8 to 10 grams of phenothiazine. A third fecal examination was made a week later, long enough after weaning (38 days) for the development of any of the common nematodes occurring in sheep, if infection had been acquired while the lambs were with the ewes. In addition to the two types of parasites found previously, one *Nematodirus* egg was found in the droppings of lamb C. This showed that despite all precautions a light *Nematodirus* infection had been acquired by one of the lambs and suggested the possibility that the other lambs possibly also harbored subdetectable infections with the same species. A second test made at the same time (3 days before four of the lambs were put on pasture) by culturing 1 to 6 grams of feces from each animal, again yielded only *Strongyloides* larvae.

4. *Methods.*—Fecal examinations were made by microscopic examination of salt flotation preparations representing one-half gram of feces.

At post-mortem examination the fourth stomach, small intestine, and large intestine were carefully searched for worms and worm lesions. Each part of the alimentary canal was then stripped separately, the contents screened through screens of coarse, medium and fine mesh, and the screenings searched carefully for worms.

The number of eggs passed per day by the sheep which contaminated the pasture was calculated from the eggs-per-gram counts and the monthly weights of the sheep, using the conversion factors established by Stoll (1929) for the appropriate fecal consistency.

5. *Weather data.*—Weather data for the three periods of the experiment are given in Table 1. These local data include figures for the monthly average, and range, of the maximum and minimum daily temperature readings made at the laboratory of the Zoological Division at the Beltsville Research Center by Mr. A. Dinaburg. The monthly rainfall was totalled from the record of daily rainfall made by the Bureau of Dairy Industry at a point approximately 1.2 miles from the experimental pasture. The conclusions drawn as to the normality of the weather conditions during the three periods of the experiment are based on the deviations from normal listed in the monthly meteorological summaries of the U. S. Weather Bureau for Washington, D. C., which is approximately 15 miles away.

## PLAN OF EXPERIMENT AND RESULTS

### I. *Contamination of the Pasture*

The pasture used in the experiment had been exposed to daily contamination by nodular worm eggs passed in the feces of from one to four sheep between May 10 and November 1, 1940. One of these sheep was a yearling which had been experimentally infected with nodular worms 3 months previously, the infective larvae having been cultured from eggs taken from adult worms. This sheep was passing

approximately 100,000 eggs each day when put on the pasture. The other three animals were spring lambs which picked up nodular worms from the pasture. One began to pass eggs at the end of July and the other two at the end of August. During the 6 months' period the 4 sheep passed a calculated total of 89,500,000 eggs onto the 0.7 acre pasture, and all showed, by gradual increase in the number of eggs passed, evidence of reinfection. All four sheep also passed numbers of *Strongyloides* eggs and coccidial oöcysts during the entire period, and in August very small numbers of *Trichuris* eggs were observed in the feces of two of the sheep, and *Capillaria* eggs in the feces of one.

Weather conditions were fairly normal during this period except that in June it was unusually hot and the rainfall markedly subnormal (Table 1). Rain fell on

TABLE 1.—*Local weather data (Beltsville, Md.) for the three periods of the experiment*

| Experimental period | Date | Air temperature (degrees F.) | | | | Precipitation | |
|---|---|---|---|---|---|---|---|
| | | Maximum | | Minimum | | Total inches | Number of days on which rain fell |
| | | Range | Average | Range | Average | | |
| I. Period of contamination | 1940 May | 51– 91 | 76 | 38–61 | 51 | 3.8 | 8 |
| | June | 72– 91 | 83 | 44–69 | 61 | 0.6 | 2 |
| | July | 74–100 | 87 | 49–84 | 64 | 3.1 | 6 |
| | Aug. | 68– 92 | 82 | 53–72 | 63 | 3.0 | 11 |
| | Sept. | 64– 90 | 77 | 36–67 | 52 | 4.3 | 7 |
| | Oct. | 45– 80 | 63 | 26–53 | 42 | 3.3 | 6 |
| II. Overwinter rest period | Nov. | 37– 70 | 53 | 21–46 | 34 | 4.9 | 7 |
| | Dec. | 28– 64 | 48 | 6–46 | 27 | 2.3 | 8 |
| | 1941 Jan. | 32– 53 | 39 | 10–33 | 21 | 3.1 | 7 |
| | Feb. | 30– 52 | 41 | 11–34 | 21 | 0.9 | 4 |
| | Mar. | 25– 62 | 48 | 12–44 | 25 | 1.7 | 5 |
| | Apr. | 56– 94 | 72 | 28–57 | 43 | 2.7 | 4 |
| | May | 59– 95 | 81 | 36–69 | 51 | 1.3 | 11 |
| III. Period of pasture test | June (2nd through 15th) | 64– 87 | 68 | 46–62 | 51 | 3.7 | 9 |

at least 40 separate days and amounted to a total of 18.1 inches, which was 4.5 inches less than normal. Conditions for the development and survival of larvae were considered good in May, poor in June, relatively poor in July and August because of depleted forage and a preponderance of hot, dry days, probably quite favorable in September, and unfavorable in October on account of the decreasing temperature. During the last three months of the period of contamination only scattered shoots of grass were left for the sheep to graze on.

## II. *Overwinter Resting of the Pasture*

The pasture was unoccupied and unentered during the 7-month period from November 1, 1940 to June 2, 1941. The weather (Table 1) was fairly normal during the winter, although the amount of snow was slightly less than usual. The temperature was slightly subnormal in February and March, and slightly above normal in April and May. From February through May there occurred a dry spell with subnormal humidity and increased hours of sunshine, and a cumulative deficiency in rainfall. However, rain fell on at least 46 days in the 7-months' period, although the total amount (16.9 inches) was 6.3 inches less than normal. Fecal pellets remained intact on the pasture throughout this period, and, until a new

growth of grass appeared late in March, the pellets were exposed to the action of sun, wind, and rain, except in the shelter and in a few places along the fences where the pellets were covered by leaves. By the end of the period a good growth of short grass had appeared again and hidden the pellets.

### III. *Test for Survival of Larvae on Pasture*

A. *Direct test for presence of eggs and larvae.*—At the end of the rest period, namely on June 2, 1941, an attempt was made to recover nodular worm eggs from the ridges of pellets found at the edges of the bare areas around trees, and from the pellets and soil. Pellets were soaked in water, crushed and examined by the usual flotation method; a few sporulated and apparently viable coccidial oöcysts were found, but nematode eggs were absent. A 19-gram sample of pellets and underlying topsoil was placed in a Bearmann funnel; only free-living nematodes were recovered.

B. *Animal test for survival of larvae.*—On June 2, 1941, two of the test lambs (A and B) were placed on the experimental nodular worm pasture and left there for two weeks with the grass as their only source of food. This interval was considered long enough to allow extensive grazing over the entire pasture and yet short enough to eliminate practically any possible chance of the lambs reinfecting themselves as the result of infection picked up on the pasture or of the presence of subdetectable infections in the lambs. Another pair of lambs (C and D) were left in the cage as controls to test the possibility of accidental acquisition of infection from ewes, stalls, or cage. The third pair of lambs (E and F), which served, when slaughtered, as additional controls on the freedom of the test lambs from accidental infection with nodular worms, were placed for the same interval on a pasture which had been contaminated the previous summer with the sheep stomach worm, *Haemonchus contortus*. The result of this test will be reported in detail elsewhere (Sarles, 1943).

Weather conditions were considered exceptionally favorable for the acquisition of infection while the lambs were on pasture (Table 1). The lambs went on pasture at the end of three days of rain which had followed two weeks of rainless weather. Consequently the ground, the fecal pellets still present from the previous year, and the grass were all soaking wet when the lambs first came in contact with them, and if any infective larvae had been present in the soil or pellets they should have had optimum stimulation to migrate from the soil or pellets onto the grass. Moreover, the weather continued to be abnormally wet, alternating between periods of several days of cloudy, wet, humid weather, and occasional days of clear weather with bright sunshine. Rain fell on 9 of the 14 days, and totalled 3.7 inches; sometimes it fell in the form of heavy showers and sometimes as a slow drizzling rain. Direct observation of the forage and of the behavior of the sheep revealed that the latter grazed in all kinds of weather, even during showers, over the entire pasture, both on short and long grass as well as on weeds.

The two pairs of lambs (A and B, and E and F) were taken off pasture at the end of two weeks and returned to the cage containing the third pair. A third fecal examination made at this time, 55 days after the lambs had been weaned, revealed in the feces of all 6 lambs only the previously detected coccidial and *Strongyloides* infections.

The final fecal examination, made after the lambs had been held in the cage for 3 more weeks to allow time for development to macroscopic size of any worms which might have been picked up from the pasture, gave the same negative result, except for one *Trichuris* egg from lamb C, the same unpastured animal which had previously shown a *Nematodirus* egg on one examination.

Lambs A and B were slaughtered 22 and 25 days, respectively, after removal from the nodular worm contaminated pasture. No nodular worms, and no nodules or other worm lesions, were found in the intestines of either lamb, and the fourth stomach of each was also free from worms. One *Nematodirus* was recovered from the small intestine of lamb A, and 3 *Nematodirus* and 8 *Strongyloides* from lamb B.

Lambs E and F were slaughtered 23 and 24 days, respectively, after removal from the stomach worm contaminated pasture. Neither showed worm lesions, stomach worms, or nodular worms, but one *Nematodirus* and 33 *Strongyloides* were recovered from lamb E, and 67 *Strongyloides* from lamb F.

Since the only species of worms found in the test sheep were small numbers of two of the three species of nematodes already known to be present in some of the test lambs as the result of accidental infection, the experiment showed that nodular worm larvae had not survived overwinter on the pasture. Because the result of the experiment was negative, and because the two pairs of pastured lambs served as controls on each other, it was considered unnecessary to slaughter the third pair of lambs at this time. Lamb C was slaughtered a year later, after having been held in the meanwhile in a cage to preclude accidental infection. At necropsy it was found to harbor 2 *Trichuris* and one *Nematodirus*. Lamb D was not killed, but during the year that elapsed before it was used for another experiment only *Strongyloides* eggs were seen in repeated fecal examinations.

## DISCUSSION

Nodular worm larvae did not survive overwinter on a pasture in Maryland under the conditions of this experiment. This result confirms the findings of Dalrymple (1904), Ransom (1906), and Swales (1940) and gives added support to the preponderance of evidence showing that the free-living stages of *O. columbianum* are not highly resistant or able to survive overwinter in northern climates.

As Swales (1940) and Gordon (1941) have already emphasized, the use of this naturally occurring overwinter sterilization of sheep pastures in conjunction with medicinal treatment has great possibilities in controlling nodular worm disease in sheep. Contaminated pastures should be rested overwinter during the period when they are of little use for grazing, to make them safe for uninfected lambs and for breeding stock which have been treated. Inasmuch as the larvae do not survive overwinter on pasture it follows that breeding stock must carry the infection over from one year to the next, and these sheep constitute, therefore, the real source of danger to spring lambs and to themselves by initiating new pasture contamination. It is, therefore, very important in the control of this debilitating infection to attempt to eliminate all residual infection from breeding animals during the winter and/or early spring by anthelmintic treatment, and then to place these animals on pastures which have not been contaminated since the last grazing season.

## SUMMARY AND CONCLUSIONS

A pasture at Beltsville, Maryland, was exposed over a period of nearly 6 months in 1940 (May 10 through October) to contamination with sheep feces containing a calculated total of 89,500,000 *Oesophagostomum columbianum* eggs. Two clean lambs grazed upon this pasture for two weeks the following June, after the pasture had been rested overwinter for a period of seven months (November through May), failed to become infected with nodular worms.

The overwinter loss of nodular worm larvae occurred on a closely cropped pasture, during a fairly normal winter and an abnormally dry spring. The weather during the period the pasture was tested for survival of larvae was unusually wet and was considered very favorable for acquisition of infection if living larvae had been present.

It is concluded that in the region of Beltsville, Md., and probably also in areas having similar or more rigorous winters, the perpetuation of nodular worm infection in sheep flocks from year to year is due to the persistence of the adult worms in the intestines of breeding sheep rather than to the survival of the free-living stages of the parasite on pastures.

For the control of nodular worm infection it is recommended that pastures be rested overwinter, and that all breeding stock be given anthelmintic treatment in the winter and/or spring before being put to pasture. This method of control should prove highly effective for the control of nodular worm infection in northern states, and should incidentally help control other intestinal nematodes of sheep.

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# NOTES ON THE GENITAL SYSTEM OF THE BIRD FLUKE, *APHARYNGOSTRIGEA CORNU* (ZEDER)

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The large intestinal fluke, *Apharyngostrigea cornu* (Zeder), is frequently met with by students of helminthology in routine autopsies of bird hosts. However, due to its greatly thickened, muscular body and the dense masses of vitelline glands, favorable specimens for making whole mounts are infrequently encountered. Therefore, probably most students seldom do more with this trematode than rather accurately guess at its specific identity, label the specimen and add it to their collection. Thus, the writers have undertaken to make a detailed study of the finer structures of the worm. At the outset it may be stated that nothing unusual in the anatomy was met with although many of the details are made clearer and the total topography of the parasite is brought up to date.

We have been rather fortunate in obtaining a large number of these parasites from various species of water birds in Mississippi, Georgia and Tennessee. With an abundance of material at hand we have been able to study the species from whole mounts and a large series of sections.

## *Apharyngostrigea cornu* (Zeder, 1800)
(Figs. 1–3)

The body is sub-cylindrical in cross section, greatly elongated in outline and shows a strong dorsal curvature. The overall measurements* are: length 6 mm; posterior body 4 mm long by 1.32 mm; anterior segment 2.20 mm long by 1.70 mm. The anterior segment is more flattened than the posterior one and is formed as a somewhat flat tube; it overlaps the posterior segment for about 200 μ ventrally.

The body is covered by a relatively thick cuticle, devoid of spines, that dips into all openings. The dorsal curvature, which is marked, is due to a slight contraction of the very well developed muscle layer (Fig. 3, M) which begins anteriorly just behind the oral sucker as a double band and passes into the hindbody. At about the level of the holdfast organ the two bands of muscles become fused into a solid band and this continues into the hindbody. At the level of the reproductive organs the band of muscles becomes dispersed into thin strands and a marginal layer that continue on to the posterior end of the caudal segment.

The oral sucker is subterminal in position and measures about 150 μ in diameter. It leads into a short esophagus that is devoid of a muscular pharynx. The two ceca are slender tubes that diverge around the acetabulum but bend ventrally into the holdfast organ. After emerging from the holdfast organ, the ceca pass into the hind-

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* Only maximum measurements are given in recording the dimensions of the various organs and structures.

body and continue posteriorly somewhat laterally and dorsally to the gonads, terminating in the area adjacent to the bursa.

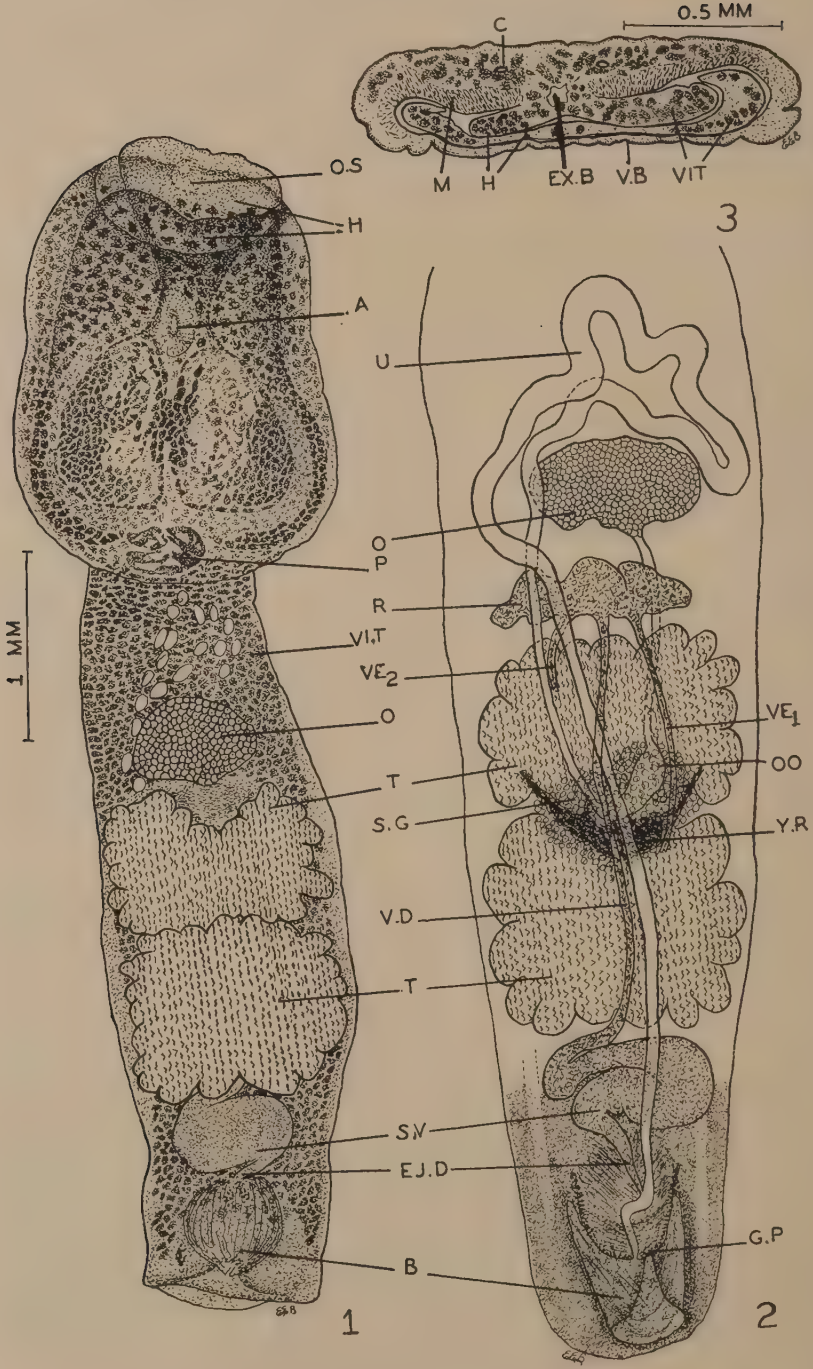
The acetabulum lies approximately 500 μ behind the caudal boundary of the oral sucker and measures 360 μ long by 240 μ wide. In whole mounts the acetabulum is difficult to make out since it is covered by the ventral wall of the anterior segment, the dorsal and ventral lobes of the holdfast organ and is usually withdrawn into a deep groove within the dorsal wall of the cavity of the anterior segment. In sections the acetabulum is seen to be definitely stalked within a groove-like receptacle.

The holdfast organ is enormously developed. It consists of two well developed lobes. The first of these develops from the base-like mass of tissue forming most of the body wall in the posterior limits of the cavity of the anterior segment. From this mass the dorsal lobe originates by an outgrowth (Figs. 1 and 3, H) into the cavity of the anterior segment. Where the lobe becomes detached from the wall two conspicuous folds remain attached to the wall for a short distance and these assist in forming the groove into which the acetabulum can be withdrawn. At about the level of the acetabulum the lobe loses its connection with the body wall and becomes a very much narrowed wedge of tissue. Anterior to the acetabulum the lobe expands again into a thin, flat, tongue-like mass of tissue and this frequently protrudes from the cavity of the anterior segment (Fig. 1, H). The ventral lobe originates from the caudal margin of the base-like mass as a broad, flat fold and follows the ventral wall of the anterior segment to the anterior end. This lobe becomes so folded as to produce a C-like tube, the lateral margins of which incloses the dorsal lobe within its cavity. The ventral lobe frequently protrudes a short distance from the cavity of the anterior segment (Figs. 1 and 3, H). Both lobes of the holdfast organ contain follicles of the vitellaria. The dorsal lobe is free of vitellaria anterior to the acetabulum but the ventral lobe is crowded with these glands to its anterior boundary.

At the base of the holdfast organ is located a large proteolytic gland. The gland consists of about six large, compact lobes which appear as clusters of deeply staining masses of cells surrounding a central vascular-like area. The entire mass may measure as much as 400 μ in diameter.

The posterior segment of the body is sub-cylindrical and strongly curved dorsally. It tapers toward both ends, being widest in the region of the testes, slightly caudal to the middle of the body. Most of the reproductive system is located within the posterior segment.

*The female reproductive system.* The transversely ovoid ovary, 460 μ long by 640 μ wide, lies across the midline in the hindbody about 650 μ posterior to the proteolytic gland. It gives rise to a long, slender oviduct which passes posteriorly along the midline, dorsally to the anterior testis to the intertesticular area. Here the oviduct becomes dilated into the oötype (Fig. 2, OO) and receives the ducts from the shell gland and yolk reservoir. The shell gland (Fig. 2, S.G) is a conspicuous mass of cells surrounding the oötype and proximal end of the uterus, and fills in most of the space between the testes. Beyond the shell gland and oötype the uterus bends ventrally and anteriorly to ascend over the ventral face of the anterior testis. In the region of the ovary the uterus becomes convoluted as it passes forward around the ovary and up to the anterior end of the posterior body segment, swinging across the body to the opposite side in ascending. From its anterior limit the uterus makes several convolutions back across the body to the same side on which it ascended,




*Apharyngostrigea cornu* (Zeder, 1800)

then descends through an almost straight course, along the mid-ventral aspect to the genital pore. In the region of the pore the uterus makes a conspicuous, right angle bend, becomes dilated somewhat and then opens into the bursa. The ova are fairly numerous within the uterus. They measure 118 μ long by 70 μ wide. The vitellaria are follicular and distributed throughout most of the body. In the forebody the vitelline glands occupy most of the area of holdfast organ and the dorsal wall of the anterior segment, being absent from the ventral wall of that segment. They reach from the level of the oral sucker to the caudal boundary of the forebody. In the hindbody the glands form a dense, ventral mass stretching across the body from its beginning to the ovary. At the ovary the vitelline glands become restricted into two narrow bands that extend down the hindbody, ventrally and laterally through the region of the testes and then fuse into a solid band in the ventral and lateral aspects posterior to the testes. They terminate just posterior to the ends of the ceca, in the area of the bursa (Figs. 1 and 3, VIT). In the area of the anterior testis, two yolk ducts converge toward the midline at the intertesticular area to form a yolk reservoir (Fig. 2, Y.R). The common yolk duct is short. It passes anteriorly from the reservoir to enter the uterus near the point where the latter emerges from the oötype.

*The male reproductive system.* The testes are large, conspicuous bodies lying in the hindbody, caudal to the ovary. They are arranged in tandem. In sections, both testes appear as bi-concave structures with their margins meeting dorsally and ventrally. In outline both testes are deeply indented to irregular. They measure 720 μ long by 1.16 mm wide for the anterior and 880 μ long by 1.20 mm wide for the posterior testis. The anterior testis is separated from the ovary by the seminal reservoir. Each testis gives rise to a vas efferens from its antero-ventral surface, one on either side of the midline (Fig. 2, $VE_1$, $VE_2$) and these pass forward, ventrally to the testicular bodies. The two vasa efferentia unite in the area between the ovary and anterior testis and form a greatly expanded seminal reservoir. The reservoir fills the space between the testis and the ovary and may extend across the entire width of body in that area. From the seminal reservoir the vas deferens (Fig.

FIG. 1. Ventral view of entire specimen (later sectioned, from which most of the sections studied were taken), showing location and relative proportions of all internal structures. Drawn with aid of camera lucida.

FIG. 2. Reconstruction of organs and organ systems in the posterior segment of the body.

FIG. 3. Cross section (drawn with aid of camera lucida) through anterior segment of body just below the level of the acetabulum, showing the wall of the forebody and the two lobes of the holdfast organ. Note the distribution of the vitelline glands in the section.

ABBREVIATIONS

A—acetabulum
B—bursa copulatrix
C—cecum
EJ.D—ejaculatory duct
EX.B—excretory vesicle
G.P—genital papilla
H—holdfast organ
M—muscle
O—ovary
OO—oötype
OS—esophagus
P—proteolytic gland
R—seminal reservoir
S.G—shell gland
S.V—seminal vesicle
T—testis
U—uterus
V.B—ventral body wall
V.D—vas deferens
$V.E_1$—posterior vas efferens
$V.E_2$—anterior vas efferens
VIT—vitellaria
Y.R—yolk reservoir

2, V.D) descends along the midline and in close proximity to the descending uterus to the area just caudal to the posterior testis. At this point the vas deferens becomes dilated and thrown into a series of folds, loops and turns, becoming more and more expanded. Then suddenly the tube becomes constricted to form a short ejaculatory duct (Figs. 1 and 2, EJ.D). The expanded portion of the vas deferens, the seminal vesicle, is filled to capacity with spermatozoa. The ejaculatory duct merges with the uterus at the angle where the latter structure makes its sharp bend before passing to the bursa.

In most whole mounts the bursa appears to be terminal although in sections it is seen to be slightly dorsal in position. The bursa is well developed and is eversible. A wide slit-like opening communicates the bursa to the outside. Internally the bursa is lined with cuticle and is surrounded by well developed muscles. A well developed genital papilla may be protruded into the bursa or withdrawn into the body. When protruded, and frequently the papilla can be seen protruding through the bursal opening to the outside, the papilla is seen to give vent to the reproductive tract, with the so called "hermaphroditic" canal (that part of the reproductive canal from the union of the ejaculatory canal with the uterus) opening at the summit. No prostatic gland cells were observed.

The above description of *Apharyngostrigea cornu* is based on a study of material taken from the little blue heron, *Florida caerulea caerulea*, from Mississippi. Other hosts from which specimens have been taken include Ward's heron, *Ardea herodias wardi*, American egret, *Casmerodius albus egretta*, eastern green heron, *Butorides virescens virescens*, and black-crowned night heron, *Nycticorax nycticorax hoactli*.

SUMMARY

The bird fluke *Apharyngostrigea cornu* (Zeder, 1800) is reported from a number of water birds, little blue heron, eastern green heron, Ward's heron, black-crowned night heron and American egret. The finer details of its internal anatomy are described and illustrated.

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# ON THE VIABILITY OF VARIOUS SPECIES OF *TRYPANOSOMA* AND *LEISHMANIA* CULTURES

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Most species of trypanosomes grow readily on original N. N. medium (3) or on its various modifications such as N. N. N. and regular blood agar slants which contain from 10% to 50% of defibrinated blood (1, 2, 4).

After the initial culture of a given species of trypanosome is obtained from vivo into vitro, the subsequent subcultures when made on N. N. medium every second or fourth week usually give luxuriant growths of trypanosomes not only in the water of condensation but also on the slant portions of the blood agar tubes (1, 2, 3, 4, 5, 6, 9).

The present study summarizes the results of the microscopic examination the stock subcultures for the viability (motility) of *Trypanosoma cruzi* and other flagellates on culture medium in vitro stored at 18°–31° C from two months to as long as six years.

## METHOD AND MATERIAL

*Cultivation and transplantation of various strains of* Trypanosoma *and other flagellates in vitro.*—From 1928 to 1942 fifteen species of trypanosomes representing over 250 strains were cultured by the writer on N. N. medium (4, 5, 6, 7, 8). Of these strains over 100 were *Trypanosoma cruzi,* some of which were isolated as early as 1932 (4). In addition to the above, four species of flagellates such as *Leishmania* and *Herpetomonas* were received from other investigators.[2] The stock cultures of the above species and strains were maintained in vitro, by monthly subculture for many generations, the minimum period for each strain in vitro being five months and the maximum over ten years.

*Storage of* Trypanosoma *and* Leishmania *cultures.*—The stock cultures of the flagellates in N. N. tubes inoculated during 1936 to August, 1941, were kept at the National Institute of Health, U. S. Public Health Service, Washington, D. C., in a room with a temperature fluctuation of from 18°–30° C. In October, 1941, these cultures were brought to Galveston, Texas, and during the winter and spring were kept at room temperatures of from 20°–27° C and during July and August of from 28°–31° C. The most recent transfer of stock cultures as a rule was kept at a constant temperature of 25° C for three months before being stored at room temperature.

*Examination for viability of trypanosomes.*—In order to ascertain the viability of trypanosomes at various periods of time, a loop of material was removed from the culture medium (usually from the water of condensation) and examined micro-

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[1] Part of this work was performed at the National Institute of Health, U. S. Public Health Service, Washington, D. C., during 1936–41.

[2] Strains of *Leishmania donovani* or *L. tropica* or both were received from Professor F. G. Novy, University of Michigan; Doctor D. Berberian, American University of Beirut; Doctor B. M. Das Gupta of Calcutta; a strain of *Herpetomonas* was received from Doctor R. W. Glaser of Princeton, N. J., and a strain of *Trypanosoma vespertilionis* from Doctor E. Dias, Brazil, South America.

scopically (objective 45×, ocular 10×) under cover-glass preparation. (To facilitate making the large number of examinations required, cover glasses 22 × 22 mm were each cut into four pieces with a diamond pencil, thus making possible eight examinations on one slide.) A majority of the experimental tubes was examined within three days; the stock cultures, on the other hand, were examined at various intervals. Usually from one to three minutes were devoted to microscopic search of each culture tube. With few exceptions the cultures which were negative by microscopic examination were not subjected to the cultural test to ascertain whether or not a scanty number of viable trypanosomes may have been present.

## DATA

The experimental data on hand show that *Trypanosoma cruzi* invariably remain actively motile on N. N. medium during the first two and even three months. Out of 520 tubes examined, 124 cultures kept in vitro for approximately six years gave 13 positive results; 290 kept from seven months to five years yielded 11 positives; and the remaining 106 kept from January to June, 1942, gave 68 positives, making a total of 92 tubes containing actively motile trypanosomes.

It is noteworthy that the contents of the tubes kept at room temperature for as long as six years were still in good condition, and the majority of the tubes even then contained 0.5 cc or more of the water of condensation. Although the formula for preparing blood agar slants (N. N. medium) was always about the same, the addition of rabbit's blood to the medium varied from 20% to 50%. This variation, together with the different lots of rabbit blood, the infusion broth, and the pH, may possibly have caused the higher percentage of positive findings as obtained from certain lots of culture tubes.

The record of the positive findings in the present study is based chiefly on actual demonstration by microscopic examination of trypanosomes in the culture tubes. In some instances when trypanosomes were not seen, positive cultures were obtained from such tubes by inoculating a few drops of the water of condensation from the N. N. tubes into new culture medium. However, this procedure was not utilized extensively in the present study. It appears that the percentage of positive findings possibly would have been higher if subcultures had been made from each microscopically negative culture tube or if more time had been devoted to microscopic search. Whenever the trypanosomes were demonstrated microscopically, the subcultures always gave positive results.

It is observed that *Trypanosoma cruzi* remain viable for longer periods on N. N. medium than do other species of trypanosomes. Based on over one hundred cultural observations, the viability of *T. avium* on blood agar slants in vitro was found to be usually one but rarely more than three months; similarly, *T. rotatorium,* about three to four months, provided the cultures were kept at a room temperature of not higher than 25° C. A limited number of subcultures of *T. americanum, T. duttoni, T. lewisi, T. melophagium, Leishmania donovani,* and *L. tropica* was found to yield negative results at the end of four months; however, in many instances subcultures were obtained at the end of two and occasionally during the third month. In one instance *Herpetomonas musca domestica* were found viable and actively motile on N. N. medium after 2066 days (about six years).

As the cultures grew older, some of the flagellates gradually became pear-shaped and rounded in form. The rosettes assumed a granular appearance in small and large aggregations, which eventually led to disintegration and death.

Fifteen species of trypanosomes and four other species of flagellates (representing over 250 strains) kept on blood agar slants (N. N. medium) were found to be actively motile when one month old. Subcultures from such tubes nearly always yielded 100% positive results.

## SUMMARY

Out of 124 subcultures of *Trypanosoma cruzi* kept in vitro for approximately six years without further subculturing, 13 tubes were found still to contain actively motile trypanosomes; 291 culture tubes kept from seven months to five years gave 11 positive results, and the remaining 110 cultures kept about six months presented 68 positive findings.

The viability of *T. avium* on blood agar slants in vitro was found to be usually one but rarely more than three months, and of *T. rotatorium* about four months when the cultures were kept at room temperature not higher than 25° C. A limited number of subcultures of *T. americanum, T. duttoni, T. lewisi, T. melophagium, Leishmania donovani* and *L. tropica* was found to yield negative results at the end of four months; however, in many instances subcultures were obtained at the end of two and occasionally during the third months. *Herpetomonas musca domestica* in one instance were found to be viable on blood agar slants after a period of six years.

Fifteen species of trypanosomes and four other species of flagellates (representing over 250 strains) kept on blood agar slants (N. N. medium) were found to be actively motile when one month old. Subcultures from such tubes nearly always yielded 100% positive results.

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# ANAEROBIOSIS AND CHOLESTEROL AS GROWTH REQUIREMENTS OF *ENDAMOEBA HISTOLYTICA*

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Since the introduction of methods for the in vitro cultivation of *Endamoeba histolytica*, the value of this procedure has been adequately demonstrated. It has been generally felt, however, that pure cultures, free of the accompanying bacteria, could be applied even more widely to problems of practical interest. The primary obstacle to the purification of *E. histolytica* cultures, that of separating the amoebae in a viable state from all living bacteria, has been overcome by several methods (Cleveland and Sanders, 1930; Rees, 1939; Meleney, Frye, Leathers and Snyder, 1940). In no case, however, was it possible to establish transferable cultures in sterile media with the bacteria-free preparations obtained. Since prompt development occurred upon the addition of bacteria, it is apparent that the association of *E. histolytica* with bacteria is functional rather than merely coincidental.

The nature of this association might be either simple or complex, and dependent upon one or more factors. The preliminary experiments of Cleveland and Sanders (1930) and our own (Meleney and others, 1940) have shown that a wide variety of sterile media, including culture filtrates and heat-killed bacteria, would not replace living bacteria as a requirement for either excystation or growth. It has also been found (Chinn, Jacobs, Reardon and Rees, 1942) that in certain cases, mixed cultures of two or three bacterial species induce better growth of *E. histolytica* than do pure cultures of the same species. These observations do not favor a simple explanation for the dependency of the amoebae upon bacteria, and the data to be presented in this report indicate that several factors are actually involved. If the number of significant factors should prove to be large, the possibility of providing a suitable combination by any empirical method obviously becomes remote. In order to simplify this situation, we have attempted to divide the study into several phases, each of which could be investigated separately. In this way we have already shown (Snyder and Meleney, 1941) that the requirements for excystation are suitable anaerobic conditions, ordinarily provided by the metabolic activity of bacteria. These conditions alone are not sufficient for continued multiplication of the excysted trophozoites, and various nutrient media did not supply the deficient factors.

In pursuance of this procedure of investigation, we have undertaken the analysis of the growth requirements of *E. histolytica* through a study of cultures containing bacteria. The object of the present report is to describe the progress made in this study to date.

All exploratory experiments were carried out with cultures of the Dobell (1931) monkey strain of *E. histolytica* (Strain NRS), but significant findings have been confirmed with six other strains. All culture media were employed in one milliliter volumes in culture tubes of 16 millimeter diameter, thus providing a large surface-

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volume ratio in order to emphasize any contrast between cultures incubated under aerobic and anaerobic conditions. Each tube of medium was inoculated with one drop of thoroughly mixed culture, thus providing large and relatively uniform inocula. Since essential growth factors might also be transferred under these conditions, at least five consecutive transfers in any medium were considered necessary to establish its suitability for the cultivation of *E. histolytica*. Cultures were examined directly through the wall of the culture tube placed on the stage of an horizontally inclined microscope, but the validity of this method was confirmed by slide examinations. Starch was provided as approximately two milligrams of completely dispersed grains of rice starch purified by digestion of the rice flour proteins with trypsin and repeated washing with water and fat solvents.

Although it appeared probable that the final interpretation of results obtained with mixed cultures of *E. histolytica* and bacteria might be open to question, such cultures might supply suggestive evidence bearing on several problems of immediate interest. On the one hand, since the media previously used for the cultivation of this amoeba are relatively complex, it was considered desirable to make available a simpler medium prepared with constituents of nearly constant composition, and if possible to determine the growth-essential factors provided by the medium. Following the observation of Reardon and Rees (1939) that coagulated whole egg supplies all of the organic growth requirements of *E. histolytica* cultures containing bacteria, we were able to replace this by an entirely liquid medium prepared by infusing coagulated whole egg in 0.8 per cent sodium chloride. This medium gave approximately as abundant growth as comparable media containing coagulated egg slants. Growth was heavier and more persistent if starch was added, but cultures could be maintained indefinitely without starch if transferred daily. This egg infusion medium served as a starting point for the analysis of the growth requirements of *E. histolytica* in the presence of bacteria, and as a control for comparison with the results obtained.

On the other hand, the probable significance of anaerobic conditions for the growth of *E. histolytica* had been suggested by the necessity of anaerobic conditions for excystation and by the fact that an essentially anaerobic environment predominates in cultures of oxygen-consuming bacteria (Rahn and Richardson, 1941, 1942) and in the usual habitat of the amoeba (the lower intestine). However, it is obvious that in aerobic cultures with bacteria, the amoeba are exposed to an environment which is neither strictly aerobic nor strictly anaerobic, and thus is difficult to evaluate. We chose to simplify this situation by imposing a strictly anaerobic environment upon the whole culture through absorption of the oxygen with alkaline pyrogallate sponges in the culture tubes. In egg infusion, growth of *E. histolytica* occurred for one or two transfers under these conditions, but never for three consecutive transfers. The failure of these cultures could not be ascribed to a direct effect of the lack of oxygen upon the amoebae, since growth in the first anaerobic culture was quite abundant. Furthermore, it was noted that the turbidity developing from the bacteria transferred with the amoebae was distinctly lessened after a few transfers under anaerobic conditions. This suggested the importance of large numbers of bacteria for the growth of *E. histolytica,* a requirement which had previously been demonstrated for *Dientamoeba fragilis* by Brug (1936). Consequently, anaerobic cultivation was again attempted, but each transfer was made, not into sterile medium, but into bacterial cultures which had been previously inoculated and incubated

aerobically for 24 hours. By this method, anaerobic cultures of *E. histolytica* could be maintained for an indefinite number of transfers. The most satisfactory bacterial culture found for this purpose was a mixed culture obtained by destroying the amoebae present in aerobic cultures of *E. histolytica*. This bacterial culture was maintained separately by daily transfers in egg infusion. Pure cultures of several bacterial species gave either negative or poor results. Heat-killed cultures were inactive.

After a few transfers under these conditions, anaerobic cultures of *E. histolytica* showed several characteristics which remained constant throughout the period of study. Multiplication was detectable after about 24 hours, and in two days the population reached a maximum roughly comparable to that found under aerobic conditions. Starch was required, probably as a source of carbohydrate, in these and in all other anaerobic cultures, and could not be omitted for a single transfer. On the other hand, it was possible to make occasional transfers to sterile medium rather than to a bacterial culture, but this could not be repeated for several consecutive transfers. Anaerobic cultures inoculated into sterile medium and incubated aerobically also failed to develop, unless a heavy inoculum of bacteria was added at the time of inoculation. In the latter case, there resulted a typical aerobic culture which could be maintained indefinitely under aerobic conditions by transfers into sterile media, indicating that anaerobic cultivation had at least induced no permanent change in the amoebae. In addition, anaerobic cultures showed a marked susceptibility to exposure to air. Slide examinations demonstrated that death and degeneration of practically all amoebae present in luxuriant cultures had occurred within one to three hours after opening the tubes. It was sometimes possible to obtain subcultures after this period, due perhaps to the presence of a few cysts. These observations indicate that bacteria in large numbers are required not only for the growth of *E. histolytica*, but even for the survival of trophozoites when air has access to the cultures.

TABLE 1.—*Analysis of minimum cultural requirements of* Endamoeba histolytica

<table>
<tr><td></td><td>"Aerobic" conditions</td><td colspan="4">Anaerobic conditions</td></tr>
<tr><td></td><td></td><td>1</td><td>2</td><td>3</td><td>4</td></tr>
<tr><td rowspan="4">Media (1.0 ml)</td><td rowspan="4">Sterile egg infusion</td><td rowspan="3">Whole, living, aerobic, mixed-bacteria culture in egg infusion</td><td>Living, washed, mixed-bacteria</td><td>Fresh, sterile cysteine</td><td>Fresh, sterile cysteine</td></tr>
<tr><td rowspan="2">Sterile mixed-bacteria culture filtrate</td><td>Sterile peptone-Ringer's solution</td><td>Sterile peptone-Ringer's solution</td></tr>
<tr><td>Sterile egg infusion</td><td>Sterile cholesterol</td></tr>
<tr><td colspan="4">Sterile rice starch</td></tr>
<tr><td>Inoculum (1 drop)</td><td colspan="5">*Endamoeba histolytica* and bacteria</td></tr>
</table>

The analysis of the growth requirements of *E. histolytica* under anaerobic conditions proceeded as indicated by Media 1 to 4 of Table 1. It was found that the activity of the bacterial culture (Medium 1) resided neither in the bacteria alone, nor in the fluid portion alone, since neither washed, living bacteria nor culture filtrate added separately to sterile egg infusion provided a suitable substratum for anaerobic cultivation. However, the activity of the culture had not been destroyed by washing or by filtration, since reconstitution of the culture (Medium 2) permitted anaerobic growth. This is a clear indication that more than one growth requirement was supplied through bacterial activity. Therefore, the two fractions were studied separately.

Washed, living bacteria could not be replaced by heat-killed bacteria, but it was found possible to substitute sterile reducing agents for this fraction (Media 3 and 4). Of the reducing agents tested to date, cysteine permitted quite as abundant growth as did the bacterial fraction, "reduced iron" gave distinctly poorer results, and thioglycollate, sulfide, sulfite, formaldehyde-sodium sulfoxylate and zinc metal were found inactive. To a certain extent, therefore, the requirements for growth parallel those for excystation (Snyder and Meleney, 1941), since in both cases elimination of oxygen and the presence of reducing agents appear to be essential. However, the requirements for growth are apparently more specific, in that thioglycollate, which had induced excystation quite as readily as cysteine, did not permit growth in the present situation. It is probable that the sole function of the accessory bacteria added as the bacterial fraction is that of reducing activity due to bacterial enzymes, but is not known whether this activity is concerned only with hastening the removal of traces of oxygen dissolved in the medium, or whether it also includes other essential oxidation-reduction reactions.

The filtrate fraction, on the other hand, apparently supplied at least two growth-essential factors, since it was replaceable only by a combination of substances. These factors could be provided by a mixture of sterile Difco Proteose-peptone in Ringer's solution and sterile egg infusion (Medium 3).

Sterile egg infusion was replaced by cholesterol (Medium 4), but the significance of this compound requires further qualification. Substitution alone is not sufficient to prove that cholesterol and the active factor provided by egg infusion are identical. However, comparison of the lowest concentration of chemically pure cholesterol permitting growth (between 1 : 1 million and 1 : 10 million) with the growth-limiting dilution of an ether-extract of egg yolk (between 1 : 50 thousand and 1 : 150 thousand of the original yolk) and the known cholesterol content of egg yolk (1 : 100 to 1 : 300) suggests that cholesterol is the active substance in egg, since these values approximate within reasonable limits. The few related substances which were tested (bile salts and non-irradiated ergosterol) were found inactive. The observations of Dopter and Deschiens (1938) on the effect of cholesterol and bile salts on the growth of *E. histolytica* were made in a medium which must have completely satisfied any requirement for cholesterol, and thus are not especially pertinent to the present study. This requirement is not entirely specific, however, since we were able to replace cholesterol by an ether-extract of rice flour. This is in agreement with the results of Cailleau (1937) who found cholesterol and certain plant sterols interchangeable as growth requirements for several species of flagellates. The sterol content of rice flour also may explain a few reports of the cultivation of

*E. histolytica* in media lacking egg yolk, blood serum or other obvious source of cholesterol (Cleveland and Collier, 1930; St. John, 1932; Tsuchiya, 1932, 1934). Our own starch preparation was treated to remove sterols, and our experiments were checked by controls to which cholesterol was not added.

Ringer's solution was used routinely, but the individual salts which it contains have not been tested to determine whether or not they are all essential. Peptone could not be omitted, but liver extract or meat extract were equally satisfactory. The significance of this fraction is not entirely clear. Superficially, it might be presumed to act merely as a source of suitable organic carbon and nitrogen compounds. If so, egg infusion must be considered deficient in these compounds until acted upon by living bacteria under aerobic conditions (Media 1 or 2), since the addition of washed bacteria alone to sterile egg infusion without a period of aerobic incubation did not provide a suitable substratum for anaerobic cultivation.

Conversely, the cysteine-peptone-cholesterol medium (Medium 4) was not found suitable for aerobic cultivation of *E. histolytica*, regardless of the type of culture employed as the inoculum, although the turbidity due to bacterial growth was not appreciably less than in aerobic egg infusion cultures. Continued aerobic cultivation was accomplished, however, by simultaneous inoculation with washed bacteria *at each transfer*. It seems probable, therefore, that the failure of the peptone-cholesterol medium in the presence of air is related in some way to the reducing activity of the culture, since this was identified as the function of supplementary washed bacteria under anaerobic conditions.

In addition to the requirements already indicated, anaerobic *E. histolytica* cultures contain a residual bacterial flora transferred with the inoculum, which is also essential for growth. The need for these bacteria is shown by our failure to obtain pure *E. histolytica* cultures in any of our simplified media by inoculation with bacteria-free cysts. The essential nature of these bacteria introduced the possibility that the growth requirements which have been described are related to these bacteria rather than directly to *E. histolytica*. An unqualified decision cannot be made on this point as yet. However, the rapidity with which death occurs in exposed anaerobic cultures is most readily explained as a relatively direct action of oxygen upon the amoebae, and our results as a whole suggest a degree of obligatory anaerobiosis on the part of *E. histolytica* comparable to that of the most sensitive bacterial anaerobes. In regard to cholesterol, it appears quite unlikely that this substance is a growth requirement of essential bacteria. Of the numerous species of bacteria found by Chinn, Jacobs, Reardon and Rees (1942) to be capable of supporting the growth of *E. histolytica* when introduced as pure cultures, many are known not to require cholesterol or related substances, at least in concentrations significant to the present study. We have obtained anaerobic cultures containing only *E. histolytica* and a single species of obligatory anaerobic bacteria isolated from the anaerobic cultures previously described. Cholesterol had no effect on this bacterium in pure culture, and is therefore presumed to be required by the amoebae, either as such, or after chemical alteration by the bacteria. This partially purified culture also required the addition of blood serum to the medium in order to obtain any growth of *E. histolytica*. Since pure cultures of the bacterium grew luxuriantly in the absence of serum, this requirement also may probably be referred directly to the amoebae. The serum factor appears to be heat-labile, but has not yet been studied further. The

presence of serum has so far prevented our testing these cultures directly for their reaction to cholesterol, but there is nothing to suggest that this requirement might have disappeared under these conditions. At least one additional growth factor, perhaps of a supplementary nature, is suggested by the fact that *E. histolytica* cultures containing only this single bacterial species produced only about half the number of amoebae found in anaerobic cultures with mixed bacterial species, and were characterized by a prolonged lag period of about six days. Some improvement was observed in the presence of liver extract, casein or additional peptone, but these results were irregular.

The growth requirements of *E. histolytica,* as far as they are known or have been suggested in the preceding discussion, are outlined tentatively in Table 2. Although

TABLE 2.—*Probable growth requirements of* Endamoeba histolytica

| | Factor | Former source | Present source |
|---|---|---|---|
| 1 | Water | | |
| 2 | Inorganic salts | Ringer's solution, egg, etc. | Ringer's solution, peptone |
| 3 | Amino acids (?) | Egg, serum, etc. | Peptone |
| 4 | Carbohydrate (?) | Rice flour | Rice starch |
| 5 | Cholesterol | Egg, serum, rice flour | Cholesterol |
| 6 | Elimination of oxygen | Bacteria | Alkaline pyrogallate |
| 7 | Other reducing activity (?) | Bacteria | Cysteine |
| 8 | Heat-labile factor | Bacteria | Serum |
| 9 | Other essential factors (?) | Bacteria | Anaerobic bacterium |
| 10 | Supplementary factors (?) | Bacteria | Liver extract, casein, etc. (?) |

they are listed as the complete or partial requirements of an hypothetical pure culture of the amoebae, it is obviously impossible to test these suggestions in an unequivocal manner until the residual living bacteria are replaced by the remaining unknown factors which they supply. Our results may, therefore, be found capable of other interpretations, but it appears desirable at the present time to regard most of the factors listed as distinct growth requirements of *E. histolytica,* and to base further study on this hypothesis.

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# SURVIVAL ON GRASS PLOTS OF EGGS AND PREINFECTIVE LARVAE OF THE COMMON SHEEP STOMACH WORM, *HAEMONCHUS CONTORTUS*

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Attempts to control the common stomach worm, *Haemonchus contortus,* have been directed chiefly toward the elimination of the parasites by the use of medicinal agents. Another method of control, namely, pasture rotation, has been advocated, but in order for this method to be successful, it must be based on a thorough knowledge of the free-living stages of the parasites and on the conditions which influence the embryonation of eggs and the development and survival of the larvae. Of the various preparasitic stages in the development of the parasite, the greatest attention has been paid to the third stage or infective larvae because they are capable of infecting sheep. However, since the infective larvae can develop only from preinfective larvae, the factors that influence the development and survival of these stages are likewise important. It is the purpose of this paper to present data on the influence of temperature, sunlight, and moisture on the survival of the preparasitic stages of *H. contortus.*

## HISTORICAL REVIEW

Veglia (1915) studied the effect of high and low temperatures, drying and sunlight on the preinfective stages of *Haemonchus contortus* and found that stomach worm eggs kept in the dark at 26° C under favorable moisture conditions reached the tadpole stage in 6 hours; 25 per cent of the eggs hatched in 14 hours, 50 per cent in 17 hours and the majority of the remainder in 48 hours. This temperature might be considered as optimum for embryonic development. According to Veglia, deviation from this optimum caused either a slowing of embryonic development or death. Some eggs kept at 50° C survived for two hours but all were dead in four hours. Eggs in feces kept at 40° C invariably failed to develop as did 2 of 6 cultures kept at 37° C. At 15° to 18° C development was normal but slowed; at 8° C larvae in fecal cultures reached the second stage. No change took place in eggs kept at 4° C

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and all eggs in feces placed in a layer between blocks of ice were dead in 24 hours. Veglia also found that when *Haemonchus* eggs in feces were placed in sunlight during summer they became devitalized within two days and in some lots in less than one day. His attempts to culture fecal pellets containing numerous eggs always failed when the fecal material was mixed with sufficient water to reduce the pellets to the consistency of pulp. Immature larvae and eggs, except when in the vermiform embryo stage, did not resist drying. Some eggs in the vermiform embryo stage apparently were able to survive in dried feces for as long as 20 days. Mönnig (1930), on the other hand, found that all preinfective stages of *H. contortus,* including the vermiform embryo, were killed by drying.

## MATERIALS AND METHODS

In the experiments reported in this paper, 4-foot square grass plots, each divided into 2-foot square subplots, were sowed with a mixture of pasture grasses and white clover. After the herbage had reached a height of four inches, feces containing eggs and larvae were placed on the subplots. When the period of experiments was completed the grass was cut and washed, and the washings were examined for infective larvae. Larvae in the soil were recovered by means of the Baermann apparatus, the first 25 cc of water drawn from the Baermann funnel after 48 hours being examined for larvae.

## EXPERIMENTAL DATA

### *Experiment 1: Survival of* Haemonchus contortus *Eggs in December, 1936, February, 1937, and March, 1937*

Grass sowed in the fall of 1936 was trimmed at weekly intervals until the onset of cold weather. Fresh feces containing the eggs were placed in the center of each subplot of two plots in December, 1936, in the subplots of a third in February, 1937, and of a fourth in March, 1937. Cultures made at the same time and from the same lots of feces used on these plots were incubated at room temperature and examined two weeks after preparation. The data of this experiment are shown in Table 1.

TABLE 1.—*Effect of winter weather on the survival on grass plots of the eggs of* Haemonchus contortus *in the feces*

| Date of preparation of subplots | Amount of feces placed in each subplot | Eggs per gram of feces | Minimum temperature in °F on 4 days after exposure | | | | Larvae recovered | | Larvae recovered from 100 grams control cultures |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | 4–28–37 | 5–6–37 | |
| | *grams* | *number* | *1st* | *2nd* | *3rd* | *4th* | *number* | *number* | *number* |
| 12–14–36 | 200 | 2,200 | 28 | 30 | 31 | 40 | 0 | 0 | 186,000 to 194,000 |
| 12–21–36 | 200 | 1,600 | 32 | 29 | 22 | 26 | 0 | 0 | 54,510 |
| 2–10–37 | 266 | 19,200 | 27 | 20 | 31 | 38 | 0 | 0 | 446,520 to 511,090 |
| 3– 5–37 | 533 | 11,200 | 42 | 37 | 34 | 38 | 0 | 0 | 926,520 to 1,208,850 |

These data show that apparently the embryos in the eggs of *H. contortus* were destroyed by exposure to cold weather. As no larvae were recovered from the 2 subplot examinations, the remaining subplots were not sampled.

### *Experiment 2: The Protection against Sunlight, Heat, and Drying Afforded the Eggs and Larvae of* H. contortus *by Grass*

On August 5, 1937, 50 grams of sheep feces containing 13,800 eggs of *H. contortus* per gram were placed on each of two subplots. Two weeks later 376 larvae

were recovered from the grass and 988 larvae from a one-inch layer of soil from one of the subplots. Just after removing the grass and soil from this subplot, 100 grams of a 4-day old fecal culture were added to each subplot. Two 100-gram samples from the same lot of feces used in preparing the culture yielded by the Baermann technique an average of 27,265 second and third stage larvae. The feces on the two subplots were exposed in the afternoon. A light sprinkle of rain fell 4 hours later, and during 6 of the succeeding 7 days there was a total rainfall of 5.39 inches. The plot was exposed to sunshine only 47 per cent of the possible maximum time, but in spite of this relatively small amount of sunlight the feces and the soil to a depth of one inch when examined at the end of one week's exposure yielded only 21 larvae. The control plot yielded 6,070 larvae, of which about 5,000 probably came from the second inoculation. This experiment shows that second and third stage larvae cannot survive in numbers on bare ground in summer even in wet cloudy weather.

### *Experiment 3: The Effect of Moisture and Consistency of Feces on the Survival of Preparasitic Eggs and Larvae*

In July, 1936, 100 grams of feces containing eggs of *H. contortus* were placed on the 20 subplots of 5 grass plots. Feces were applied in a different manner to each plot as shown in Fig. 1. Twelve 100-gram control cultures kept in the laboratory until the plots were examined each yielded from 434,000 to 1,031,000 larvae.

Three of the subplots (a, b, and c) of each plot received water applied with a sprinkling can. Subplots "a" of each plot received one quart of water daily in addition to whatever rain fell, subplots "b" one-third of a quart daily, while subplots "c" each received one-third of a quart of water at weekly intervals. The water added to these subplots during the 18 days the experiment lasted was approximately equivalent to 1.8, 0.54 and 0.07 inches of rain, respectively. Subplots "d" received no water other than that due to rainfall. Rain totalling 0.47 inch fell on 4 of the 5 days previous to the test period, while on 9 days of the period of experiment the rainfall amounted to a total of 3.5 inches. The temperature ranged from 60° to 90° F, with a daily mean of 69° to 86° F. The sun was shining an average of 65 per cent of the possible maximum time.

The number of larvae washed from the grass cut from the 20 subplots is shown in Table 2.

TABLE 2.—*Effect of condition of feces, of manner of their distribution on soil surface, and of moisture on survival of eggs and larvae*

| Feces applied to subplots as* | Distribution of feces on soil surface | Amount of water added to subplots after feces were applied | | | | Total larvae recovered |
|---|---|---|---|---|---|---|
| | | 1 qt. daily | 0.33 qt. daily | 0.33 qt. weekly | None (control) | |
| | | Larvae recovered from grass cut from subplots 18 days after the feces had been applied | | | | |
| | | *number* | *number* | *number* | *number* | *number* |
| Whole pellets | Scattered over area | 20,856 | 246 | 706 | 59 | 21,867 |
| | In a pile in center of area | 7,212 | 714 | 1,150 | 259 | 9,335 |
| Crushed pellets | Scattered over area | 5,018 | 1,648 | 1,512 | 345 | 8,523 |
| | In a pile in center of area | 14,701 | 12,075 | 2,228 | 420 | 29,424 |
| Watery paste | In center of area | 15,558 | 6,464 | 7,050 | 1,565 | 30,637 |
| Total | | 63,345 | 21,147 | 12,646 | 2,648 | |

* The amount of feces in all cases was 100 grams.

As the larvae of *H. contortus* under favorable conditions reach infectivity in 4 or 5 days, it may be noted that this experiment tested not only the survival of pre-infective larvae and eggs but the survival of infective larvae as well. The difference between the number of larvae recovered from the grass and the number recovered from the control cultures represents the number of larvae left on the grass stubble, in the debris and feces on the soil surface, and in the soil, as well as the eggs and

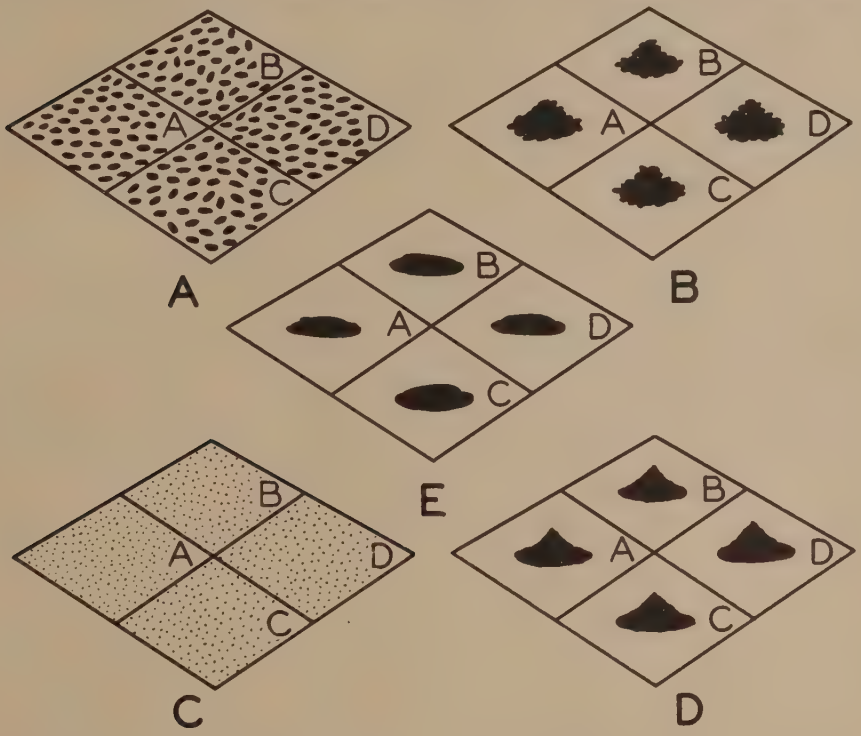


FIG. 1. Diagram showing distribution of sheep feces containing eggs of *H. contortus* on experimental plots.

A: feces in the form of pellets spread evenly over subplots; B: feces in the form of pellets piled in center of each subplot; C: pulverized feces spread evenly over subplots; D: pulverized feces piled in center of each subplot; E: feces reduced to fluid paste with an equal quantity of water and poured onto the center of each subplot.

larvae that failed to survive. The difference between the numbers of larvae recovered from the different subplots represents the total effect of the detrimental influences on the different subplots, and indicates that under natural conditions the destruction of eggs and larvae of the stomach worm is extensive. Concentration of fecal material or addition of water protects the preparasitic stages and allows larvae which otherwise would have perished to reach infectivity.

## DISCUSSION

The three experiments reported in this paper were undertaken in order to secure information on the survival of eggs and preparasitic larvae of *Haemonchus contortus*

in feces, when exposed to conditions prevailing on grass plots. The first experiment showed that no larvae were recovered in the spring from sheep feces containing eggs or from grass surrounding feces deposited on grass plots during cold weather. As infective larvae can survive cold weather during the entire winter (Shorb, 1943) it follows that the contaminative material present was destroyed by cold when in the preinfective larval stages or as eggs. This being true, it likewise follows that eggs of *H. contortus* in any sheep feces which may be deposited on pasture during the late fall, winter, or early spring, will be destroyed by cold unless there should be an ensuing warm period sufficiently long for infective larvae to develop from the eggs. Such periods of mild temperatures are rare during the winter in temperate climates and, therefore, infected lambs or sheep can add little in the way of stomach worm contamination to the pasture during the cold weather, making pasture rotation during this period unnecessary.

The second experiment showed that summer sunshine and heat were highly lethal to eggs of feces not protected from weather. Although during the period of test much more than the normal amount of rain fell in the region where the work was done than was normal for the time of year, only 21 larvae were recovered from the bare ground, while about 5,000 larvae survived the same conditions when protected by grass 4 inches tall. It is not unreasonable to assume that even 21 larvae could not have survived had there been no rain or even if only the normal amount for the season had fallen. However, it is important to remember that even on bare ground a few larvae survived and reached the infective stage. These results seem to indicate that although overgrazing is a detriment to pastures, it contributes to the destruction of vast numbers of *Haemonchus* eggs and larvae by exposing them to sunlight. The reverse would likewise be true, that adequate grass cover favors survival of such eggs and larvae and emphasizes the need under good pasture conditions for rotation and treatment of infected animals.

When the feces from infected sheep were placed on grass plots and no additional water added, a certain proportion of the eggs survived and the resultant larvae developed to infectivity. By modifying (adding a quart of water daily to each 4 square feet of sod) 24 times as many larvae survived and were recovered from the grass. A smaller addition of water, whether applied daily or weekly, was reflected in a smaller increase in number of survivors. The increase in number of larvae surviving appeared to be due to the constant presence of water rather than to the increased amount. A modification in the consistency of the feces or especially in the concentration of fecal material also caused a modification of the number of eggs and larvae surviving until the infective stage was reached. Obviously the increase in number of infective larvae recovered from plots having feces in piles, or having feces reduced to paste by the addition of water, was due to conservation of moisture. One exception was noticeable in the subplot where the feces were scattered as pellets and the pellets received one quart of water daily. The reason for this exception is not known. The significance of this experiment, when the data are used for planning pasture usage, is in pointing out the danger of wet seasons and concentration of manure on pasture. The lambs should be so managed that there would be no undue concentration of feces around feeding or watering places, and they should be moved to clean pastures following unusually wet periods.

The effect of adding water to fecal cultures and to fecal material on grass plots presents an interesting contrast. As Veglia (1915) noted, and this observation has

been verified by other workers as well as by the present writer, the addition of water to fecal material in glass containers for the purpose of allowing larvae to develop actually caused the culture to fail if enough water was added to make the fecal material wet. On the other hand, the addition of water to the fecal material in the third experiment for the purpose of reducing the feces to a fluid paste not only failed to reduce the number of larvae which survived but actually favored their survival. This leads to the conclusion that if the essential difference between diarrheic feces and normal pellets is one of water content, diarrheic feces during the summer actually favor survival of preinfective eggs and larvae.

SUMMARY

1. No larvae of *H. contortus* were recovered from grass plots in the spring following the application of feces containing eggs at 4 different times during the previous winter.

2. Sunlight and heat were highly lethal to the second and third stage larvae of *H. contortus* exposed in feces on bare ground in August, in spite of heavy rains.

3. A 24-fold increase in larvae of *H. contortus* recovered from 2-foot square grass plots, compared to control plots, followed the daily application by means of a sprinkler of one quart of water, although both test and control plots received twice this amount of water in the form of rain during the 18 days of test. The difference in yield was undoubtedly due to the fact that the sprinkled water was more constantly present than that from rain. The concentration of feces conserved moisture present and this was reflected in the number of surviving larvae recovered.

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## A REDESCRIPTION OF *POLYMORPHUS OBTUSUS* VAN CLEAVE, 1918 (ACANTHOCEPHALA)

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Van Cleave (1918) first described briefly the acanthocephalan, *Polymorphus obtusus*, from the water-turkey, *Anhinga anhinga*, and later (1925) identified as *P. obtusus* a specimen from the great blue heron, *Ardea herodias*. No subsequent description of this species has been found. Fourteen acanthocephala identified as *P. obtusus* have been recovered from the intestine of the ruddy duck, *Erismatura jamaicensis*. Since the writer has observed a number of morphological details not included in Van Cleave's papers, it seems desirable to extend the description of this species.

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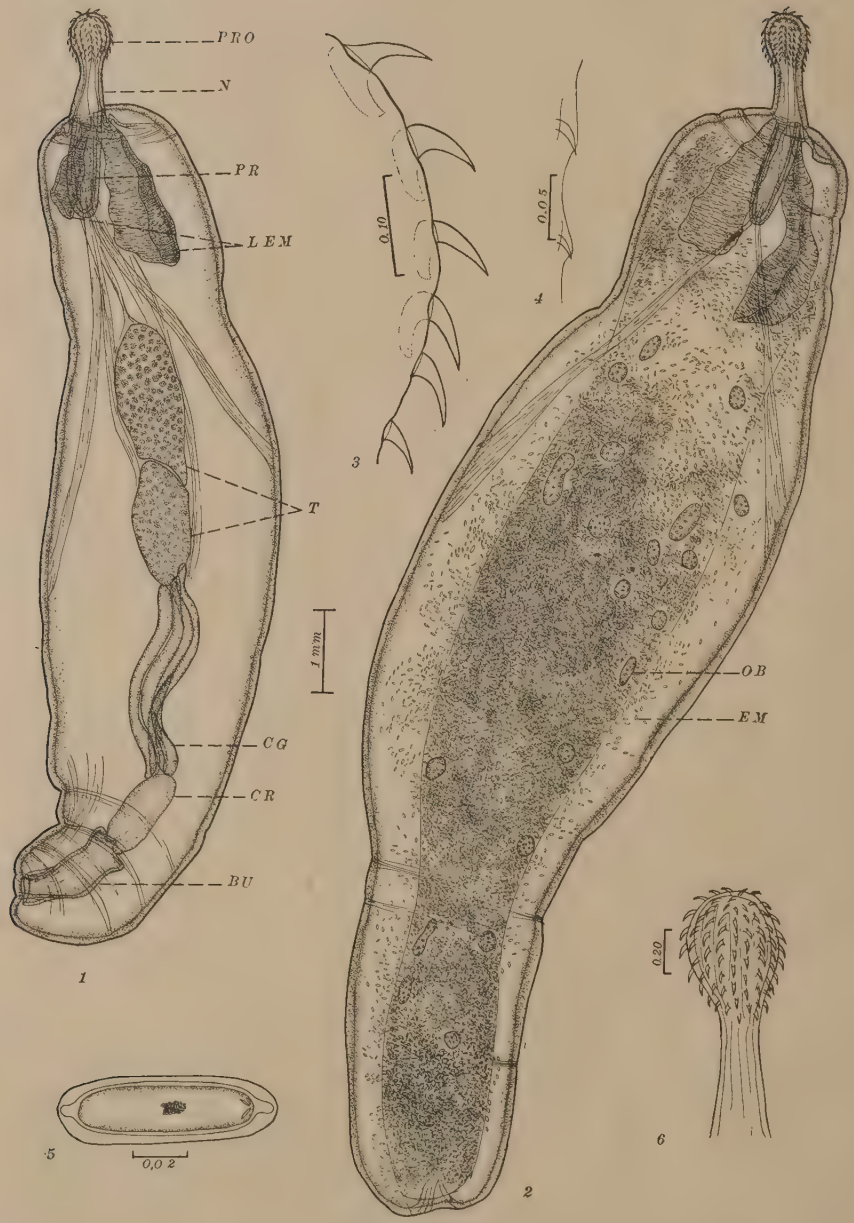


*Polymorphus obtusus*

The writer acknowledges her indebtedness to Dr. R. M. Cable for the use of laboratory facilities of Purdue University.

*Polymorphus obtusus* Van Cleave, 1918
(Figs. 1–6)

Body cylindrical with constrictions near anterior and posterior ends; posterior end truncate. Mature males (Fig. 1) 8–12 mm long by 1–3 mm in maximum diameter; mature females (Fig. 2) 12–16 mm in length by 2–4 mm in maximum diameter. Proboscis (Fig. 6) elongate ovoid, 0.5–0.7 mm long by 0.26–0.40 in maximum diameter; armed with 14–16 longitudinal rows of 7–10 hooks each. Hooks at base of proboscis 0.049–0.062 mm long; other proboscis hooks 0.066–0.073 mm long with roots 0.066–0.079 mm in length. Neck attenuate, approximately 1.3 mm long and 0.3 mm wide. Anterior part of body covered with spines (Fig. 4) about 0.027 mm long, arranged in 30–40 longitudinal rows. Proboscis receptacle 1.12–1.54 mm in length and 0.24–0.39 in diameter. Lemnisci broad, fan-shaped, with irregular margins, and unequal in size, the larger being about twice as long as the proboscis receptacle. Testes of mature males oval, 0.77–1.5 mm long and 0.56–0.77 mm wide. The four cement glands are long and tubular, averaging 2.5 mm by 0.2 mm. Fully developed embryos (Fig. 5) of mature females 0.066–0.08 mm by 0.019–0.023 mm, with polar prolongations of the middle membrane.

The writer's specimens are considerably larger than those described by Van Cleave, show greater variation in the number of longitudinal rows of proboscis hooks, and have slightly larger body spines. These differences probably are not significant, since the two lots of material are from different hosts, and the writer's specimens, being more numerous than Van Cleave's, would be expected to show a greater range of variability.

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## Explanation of Plate

Drawings were made from stained whole mounts with the aid of a camera lucida. Figs. 1 and 2 are composite drawings.

Fig. 1. Adult male.
Fig. 2. Adult female.
Fig. 3. Proboscis hooks from a single longitudinal row.
Fig. 4. Body spines.
Fig. 5. Embryo from a mature female.
Fig. 6. Proboscis of the specimen shown in Fig. 1.

BU—bursa
CG—cement glands
CR—cement receptacle
EM—embryo
LEM—lemnisci
N—neck
OB—ovarian ball
PR—proboscis receptacle
PRO—proboscis
T—testes

# CULTIVATION OF *NYCTOTHERUS CORDIFORMIS*

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Attempts to cultivate *Nyctotherus cordiformis* heretofore have been unsuccessful. The conclusion has seemed justified that this organism cannot be grown in vitro using the methods and media ordinarily successful with intestinal protozoa: indeed the opinion was expressed by Wichterman (1936) that *Nyctotherus cordiformis* is anaerobic. In the present study a method was found whereby this organism can be cultivated in an ordinary medium and without the application of anaerobic technique. The cultivation method employed is an adaptation of an intestinal content medium method previously devised (Nelson, 1940) for the cultivation of *Balantidium coli*.

## MATERIALS AND METHODS

Adult Leopard frogs, *Rana pipiens*, caught in the field or obtained from supply houses were used as the source of *Nyctotherus* throughout this study.

Preliminary attempts to cultivate *Nyctotherus* gave very encouraging results and provided a basis for the technique finally employed. In these preliminary trials the rectal contents of a frog infected with *Nyctotherus* were removed as a lump from the rectum and dropped into 10 ml of frog-Ringer's solution in a test tube. A small mass of rice starch was dropped at the side of the lump of rectal material. Careful examination with a hand lens showed that in about an hour the ciliates began to emerge from the rectal material, congregate at the starch mass, feed and then disperse. They did not return to the rectal material but migrated outward along the inside surface of the tube. For the most part the organisms spent their time crawling around on the surface of the glass.

In crude cultures of this type the ciliates lived for over a month. Morphologically and physiologically they appeared perfectly normal and some multiplication took place.

Preparatory to additional experiments an assembly was devised to (1) prepare rectal content cultivation medium, (2) separate the nyctotheri from the mass of rectal material and (3) permit removal of the mass of rectal debris from the cultivation tube. This "extraction assembly" is shown in Fig. 1. Sterile frog-Ringer (NaCl 6.5 gm, $NaHCO_3$ 0.20 gm, KCl 0.14 gm, anhydrous $CaCl_2$ 0.12 gm, $NaH_2PO_4$ 0.01 gm, $H_2O$ 1000 ml) was added to a depth of about 3 cm above the absorbent cotton in the extractor before inoculation.

Inoculation and operation of the assembly is as follows: a frog is opened, the rectum removed and the contents taken up in a large-bore pipette and introduced into the extractor over the cotton. The rectal material gives off fluid and fine solid material which diffuses and settles through the cotton into the fluid below to produce rectal content cultivation medium. The nyctotheri in the rectal material migrate downward through the cotton to the bottom of the test tube. Their movements can be followed readily with a 10× hand lens. The left-over rectal debris remains in the extractor above the cotton and when desired is removed by simply lifting out

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the extractor. There remains the test-tube of debris-free rectal content cultivation medium in which to continue cultivation of the nyctotheri obtained.

## RESULTS

The first experimental series was run at the Mt. Desert Island Biological Laboratory in Maine.

On July 16 an extraction culture was inoculated with the rectal contents of a small specimen of *Rana pipiens* caught in a near-by marsh. Within a few minutes after

FIG. 1. Extraction assembly. The extractor tube (A) is prepared from glass tubing with a thin cotton plug placed above the constriction. This tube is introduced into a test tube of medium to produce the extraction assembly (B). Material from which it is desired to separate protozoa is introduced into the extractor tube over the cotton plug. The protozoa migrate through the cotton to the bottom of the test tube. When desired, the extractor with the unwanted material can be lifted out.

the introduction of the inoculum nyctotheri were observed migrating into the cotton plug. For 45 minutes they remained in close proximity to the inoculum during which time visible downward diffusion and sedimentation from the inoculum progressed, and then they continued their migration to the bottom of the tube.

The subsequent history of the culture is as follows: at nine hours the nyctotheri were congregated at the bottom in an actively moving swarm. A small amount of

rice starch was fed and within twenty minutes the organisms became engorged and the swarm dispersed. Instead of swimming freely the ciliate was observed to be, habitually, a crawler. It crawled up the inside of the tube in a peculiar hypotrich-like manner and was able to cling with such tenacity as not to be dislodged through ordinary handling of the tubes. A count with a hand lens showed that there were about sixty-five nyctotheri in the culture.

At 22 hours the population had increased to about 120 and a number of dividing individuals were seen. Although most of the ciliates were crawling on the surface of the glass near the starch, many had crawled up the inside of the test tube, some to within a centimeter of the surface of the liquid.

At 48 hours the extractor tube with the inoculum was transferred to a new tube of Ringer's to produce a second tube of medium (Culture No. 2). Some nyctotheri clinging to the extractor accompanied the transfer. About 160 organisms remained in Culture No. 1. During the rest of the life of the culture starch was fed every three or four days.

At 10 days the population in Culture No. 1 had reached about 280 after which there appeared to be little or no increase, although the organisms apparently remained normal in appearance and activity.

On the 30th day the culture suddenly succumbed to a strange great bacterial growth following a starch feeding.

Culture No. 2 was given a slightly different treatment. At ten hours the extractor, with its contents and some clinging nyctotheri, was shifted to a new tube of Ringer's solution (Culture No. 3). About 40 nyctotheri remained in Culture No. 2. This culture was maintained for 54 days during which numbers of nyctotheri were removed for use in other experiments. The population at intervals and the numbers removed were as follows:

115 on 3rd day, 30 removed
180 on 5th day, 50 removed
420 on 30th day, 100 removed
370 on 35th day, 50 removed
400 on 38th day, 100 removed
480 on 44th day, 80 removed
420 on 54th day, 100 removed

This was a very healthy culture throughout the period of observation but it had to be terminated on the 54th day.

Culture No. 3 did not respond as well as the first two and this seems to have been due to the poor quality of the medium, as the beneficial extracts in the inoculum had been largely removed in the first two cultures.

The culture started with a population of 32 and increased slowly to about 140 on the 8th day. About 60 nyctotheri were removed on this day for use in other experiments. The loss was never entirely made up and after the 34th day the culture declined, and no living nyctotheri were seen after the 52nd day.

A second experimental series was run during the next winter in the laboratory at the University of Maine. The inoculum was taken from frogs purchased from a supply house.

On February 8th an extractor was inoculated with the rectal contents of a large frog. At the end of 24 hours approximately 60 nyctotheri were found to have descended from the inoculum. Starch feedings were given every four or five days during the life of the culture. The extractor tube was left in during the whole period. The population increased slowly to about 200 on the 15th day, about 250 on the 40th day and to a peak of about 300 on the 66th day. Thereafter a decline set in and by the 80th day the number had dropped to about 60. Only 12 active organisms and many cysts were found on the 101st day. A small amount of distilled water was added to make up for the evaporation but did not revive the culture and on and after the 105th day no active organisms were found.

A second extraction culture of Ringer's solution was inoculated on Feb. 9th with the rectal contents of a frog which had died of "red leg." At the end of 24 hours 74 nyctotheri were found to have descended from the extractor tube. The extractor tube with the inoculum was removed on the sixth day. As in the previous instances multiplication progressed slowly and steadily and by the 40th day about 270 nyctotheri were present. A slight decrease was noticed from then to the 47th day. To make up for water evaporation a small amount of distilled water was added. This had a marked stimulating effect and by the 51st day a sharp increase to about 340 organisms took place. After this date encystation began and the population dropped steadily. On the 100th day only 44 active ciliates were present. A small amount of distilled water was added. On the 105th day only three active ciliates were present and none were seen on and after the 111th day. A dilution count showed about 600 cysts to be present in the culture. These cysts appeared perfectly normal.

A third extraction culture inoculated on March 27th gave essentially the same results as the others. The extractor tube and inoculum were removed on the seventh day. Starch feeding was delayed until the seventh day by which time most of the nyctotheri had starved to death. The remaining organisms responded immediately to the starch feeding and a vigorous culture resulted. From an initial population of 23, the colony increased to 130 in 11 days, to 370 in 32 days and remained at approximately this level until the 53rd day when the population began to decline. Only 29 organisms were present on the 71st day and none on the 76th day. Excessive water loss from evaporation may have accounted for the shortened life of this culture.

*Cultivation attempts in plain Ringer's solution.*—Growth in plain Ringer's solution was attempted to determine to what extent the success of the extractor method was due to extracted material from the inoculum. Eight trials were made. In each, 15 to 40 nyctotheri were transferred to test tubes of Ringer's solution and starch was given. No multiplication took place and the organisms lived only two to four days.

*Cultivation attempts in frog blood-Ringer's solution.*—Attempts to grow the organisms in frog blood-Ringer's solution proved almost equally unsuccessful. In three attempts tubes of the medium were inoculated with from 50 to 100 organisms. A number of reinoculations were made when the organisms were found dead in two or three days. In one instance the ciliates lived for 22 days but showed lack of vigor and no apparent multiplication.

*Cultivation in pig cecal content medium.*—In pig cecal content medium (Nelson, 1940) *Nyctotherus* was cultivated for periods up to 86 days in individual tubes and by transfer for a total of 157 days, when the culture was voluntarily terminated. Cul-

ture No. 1, a test tube of 10 ml of three-month-old pig cecal medium, was inoculated with 50 nyctotheri from a 23 day old extraction culture. Growth and multiplication proceeded without delay. The population increased to about 400 by the 20th day and then slowly to a peak of about 550 on the 61st day. A decline then set in, possibly due to excessive loss from evaporation. By the 76th day the population had dropped to 56 and after the 86th day no live organisms were seen.

A subculture of 6 nyctotheri in a tube of the same medium was made on the 76th day. In three days the population had increased to 13. At the end of 23 days the population had increased to about 180 after which it increased slowly to about 300 by the 68th day. Thereafter the population began to decline with most of the organisms apparently encysting and after the 81st day no active organisms were seen.

## DISCUSSION

The writer has been unable to reconcile the statement that *Nyctotherus* is anaerobic (Wichterman, 1937, p. 567) with the results obtained in this study. No apparent need for anaerobic conditions was noted; in fact the organism was not loath to ascend to the region near the exposed surface of the culture medium and did so without suffering any adverse effect. Of course the unsuccessful cultivation attempts or other data on which Wichterman has based his conclusions are not strictly comparable. He obtained his *Nyctotherus* from *Hyla versicolor* and in the present study *Nyctotherus* from *Rana pipiens* was used. It remains to be seen whether the difference in origin may account for the difference in results. As far as is known at the present time the same species of *Nyctotherus* is present in both frogs.

The ordinary criteria of successful cultivation were satisfactorily fulfilled in the present study. Feeding, growth and normal multiplication took place and morphologically and physiologically the ciliates appeared normal. The continuous cultivation period of any single strain was only 180 days (with subcultures on the 23rd and 76th days) but there was good assurance that cultivation could have been continued indefinitely. Individual cultures lived as long as 105 days.

Opportunity was not afforded for detailed observations to determine to what extent the whole life-cycle as outlined by Wichterman (1937) was completed. Wichterman found that in the host, conjugation of *Nyctotherus* takes place only during the period of transformation of the tadpole to the frog, a period of critical changes in the animal and in the intestinal environment of *Nyctotherus*. Cultivation of *Nyctotherus* provides an opportunity to investigate in vitro the factors involved in this interesting phenomenon.

Certain of the basic requirements of the organism became apparent during this study. In plain Ringer's solution the organism does not grow or multiply and it dies in a relatively short period. The addition of rectal content extract converts Ringer's solution into a medium in which life proceeds normally until the organism dies from starvation. Starch added to this medium adequately meets nutritional requirements to promote growth and multiplication. Of the basic unknown supplements so well provided by the intestinal content extract in the medium, the bacterial population may be of critical importance in the cultivation of *Nyctotherus* as was found to be true in the case of *Balantidium* (Nelson, 1940). Serum supplement is not needed in the medium.

The successful growth of *Nyctotherus* obtained in the tests made with pig cecal content medium would seem to indicate a similarity in the requirements of *Nyctotherus* and *Balantidium*. Since both pig and frog supply, in their intestinal content, constituents essential to the growth of *Nyctotherus* it is possible that the constituents are the same in both instances. The possibility suggests itself that *Nyctotherus* could live in an animal like the pig if the temperature difference could be overcome. A number of unconfirmed reports of *Nyctotherus* infection in man and other warm blooded animals have been published. Conversely there are a number of species of *Balantidium* described from frogs.

By comparison with ordinary standards of multiplication rate and population peaks in cultures of parasitic protozoa, *Nyctotherus* is disappointing. Multiplication is deliberate, apparently amounting to only one or two divisions per 24 hours in the fresh culture and dropping steadily below this as the population increases and the culture ages. The population peak reached in a culture was about 550 nyctotheri. The total production of cultures from which organisms were withdrawn at intervals considerably exceeded this peak number.

Encystment, a rather infrequent occurrence in cultures of parasitic protozoa, took place in some of these cultures as they aged.

## SUMMARY

1. *Nyctotherus cordiformis* from *Rana pipiens*, apparently not hitherto successfully grown in vitro, has been kept under continuous cultivation for periods up to 180 days and then terminated voluntarily with good assurance that cultivation could have been continued indefinitely. Individual cultures lived as long as 105 days.

2. The organism was grown in Ringer-rectal content extract medium and fed with starch. Serum supplement to the medium was not needed. Ordinary cultivation methods were used and no indication was found that the organism needs anaerobic conditions.

3. A method designated as the "extraction culture" method was devised for separating the organisms from the rectal contents and at the same time preparing a tube of the medium.

4. The usual criteria of successful cultivation were satisfactorily fulfilled. Feeding, growth and multiplication proceeded normally and the ciliates appeared normal morphologically and physiologically. The organism seems to multiply slowly and the peak population reached in a culture was about 550. Encystment took place in some cultures as they aged.

5. Cultivation was successfully obtained in pig cecal content medium also. *Nyctotherus* appears to be similar to *Balantidium* in its requirements.

## REFERENCES

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# RESEARCH NOTES

## THE PREVALENCE OF PINWORM INFECTION IN AN OHIO INSTITUTION FOR CHILDREN

Many surveys have been made to determine the prevalence of infection with *Enterobius vermicularis* in both institutionalized and non-institutionalized groups. The more recent surveys in which use has been made of special diagnostic methods related to the characteristic ovipositing habits of the gravid female have shown that the prevalence of infection is much higher than formerly believed. Recently, the results of many surveys as well as other literature pertaining to the parasite have been summarized adequately by Cram (1941. An Introduction to Nematology, edited by J. R. Christie. Sec. II. Part II: 322–324). The present report is submitted as an addition to the existing data on the prevalence of oxyuriasis in institutionalized groups in the United States.

From April 4 to May 16, 1939, anal swab examinations were made on a group of institutionalized white children near Alliance, Stark County, Ohio. The group included both males and females, ranging in age from 3 to 18 years. Most of the children had been in the institution for more than three months, so that little effort was made to ascertain from what type of home each individual had come. The results of the examinations are given in the accompanying table.

In general, the living conditions in the institution were good. The children were divided by sex and roughly by age into six groups, each of which lived in a separate dormitory. All buildings were clean and sanitary facilities were excellent. The children slept in separate beds; however, daily bathing was not required, and the clothing was usually worn for several days before being laundered. It was possible, therefore, that ova might be carried about for several days on the bodies and in the clothing of the children. This condition probably favored the maintenance and spread of the infection.

The NIH swab devised by Hall (1937, Am. J. Trop. Med. 17: 445–453) was used throughout this survey and the finding of a single ovum on the swab was considered indicative of infection. Because of the institutional routine, most of the examinations had to be made after the morning meal. Certain infected individuals may have removed the ova from the perianal region by defecation or bathing before the examination was made, so that the number of positives may have been slightly higher than shown by the examinations. Those individuals who were positive by the first swab series were not re-examined. Of the 192 individuals who were negative by the first swab series, 125 were examined a second time.

Of the 158 males examined, 72 or 46% were infected and of 111 females examined, 34 or 31% were positive. This difference is largely due to high percentage of infected males in dormitory 3 (75%). Grouped arbitrarily by age, the rates of infection were as follows: 3–4 years, 50% (2 out of 4); 5–9 years, 65% (39 out of 60); 10–14 years, 41% (55 out of 133); and 15–18 years, 14% (10 out of 72).

TABLE 1.—*NIH swab examinations of 269 institutionalized white children*

| Dormitory and age | Sex | No. exam. once | No. pos. 1st swab | No. exam. twice | No. pos. 2nd swab | Total no. exam. | Total no. pos. | % pos. |
|---|---|---|---|---|---|---|---|---|
| No. 1 13–18 | M | 37 | 5 | 0 | 0 | 37 | 5 | 14 |
| No. 2 12–15 | M | 42 | 12 | 30 | 4 | 42 | 16 | 38 |
| No. 3 8–13 | M | 48 | 25 | 22 | 11 | 48 | 36 | 75 |
| No. 4 3–9 | M | 31 | 7 | 23 | 8 | 31 | 15 | 48 |
| No. 5 4–13 | F | 51 | 18 | 32 | 4 | 51 | 22 | 43 |
| No. 6 12–18 | F | 60 | 10 | 18 | 2 | 60 | 12 | 20 |
| Total | | 269 | 77 | 125 | 29 | 269 | 106 | 39 |

Of the 269 children examined, 77 or 29% were determined positive by the first swab series (Table 1). Of the 125 individuals who could be examined a second time, 29 were found to be positive. Thus, 106 or 39% of the 269 children examined were found infected after the second swab series. This increase in the number of infections determined by using repeated swab

series has been noted by several investigators. It seems likely that more infected individuals would have been determined in this institution if additional swab series could have been made.

The figures in Table 1 show a lower incidence of infection in the children examined from dormitories 1 and 6. This difference may be partially explained on the basis of the number of positive individuals determined by second examinations. Of 110 individuals in dormitories 2, 3, 4, and 5 who were negative by the first examination, 107 were examined a second time, whereas of 80 individuals in dormitories 1 and 6 who were negative by the first examination, only 18 could be examined a second time.

The percentage infection (39) of this group of institionalized children is considerably lower than the average percentage infection (63) for institutionalized persons of six localities in North America as summarized by Cram (loc. cit., Table 11). This difference may be explained partially on the basis of the number of swab series used in making the survey, since in most of the surveys summarized by Cram 4 to 7 swabs per person were used as compared with 2 per person in this study.—ALAN W. DONALDSON, *Department of Parasitology, School of Hygiene and Public Health, Johns Hopkins University, Baltimore, Maryland.*

## A LEECH FEEDING ON LIGULA

On July 16, 1941, a leech was discovered in the act of feeding on a live ligula along the shore of Wolf Lake, Huntington Forest property of the Roosevelt Wildlife Forest Experiment Station, Newcomb, N. Y. When placed in formalin the leech endeavored to disgorge the ligula, but succeeded in releasing only a small portion before succumbing. The uneaten portion of the worm was 160 mm long and 4 mm wide (see Fig. 1).

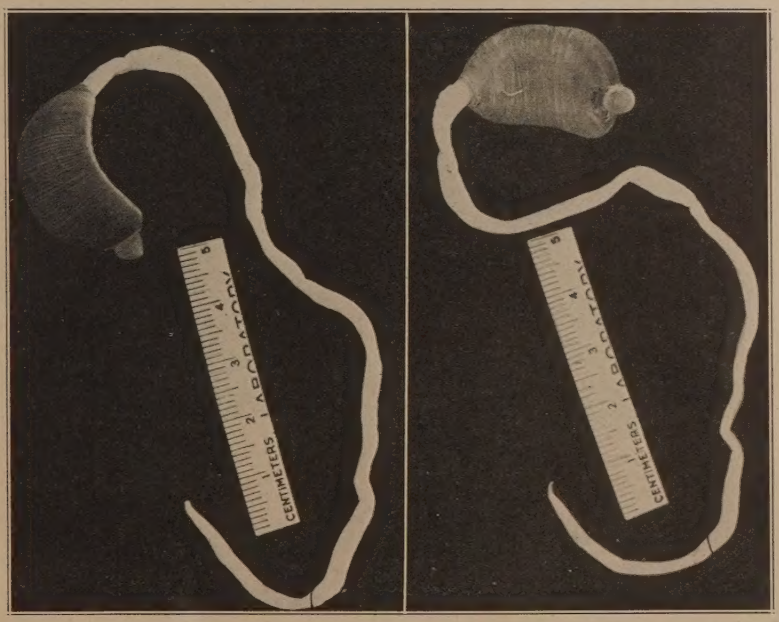



FIG. 1. Leech feeding on ligula. Dorsal view at left, ventral view at right. Photograph by J. F. Mueller.

Ligulas are sometimes found free and alive in the shallow shore areas of at least two lakes on the Huntington Forest (Dence, 1940, Copeia, **2**: 140), where about one-fourth of the large population of common shiners (*Notropis cornutus*) are infected with ligulas. Badly infected individuals, with distended bellies, commonly frequent the shallow sandy shore areas throughout the summer. It is presumed that some of the parasites are liberated by the predators, such as mergansers and herons, during the feeding process, although there is evidence that worms are

occasionally freed by other means also. The ligula that was attacked by the leech was one of several at the mouth of a little inlet.

This appears to be the first record of a leech feeding on ligula, probably because free ligulas are never very abundant. Leeches were not abundant in Wolf Lake either. Many of our fresh water leeches are predacious and regularly feed on small aquatic animals.

The species of leech has not been positively determined, but Dr. J. Percy Moore, after examining photographs, stated that it was undoubtedly a species of *Haemopis,* probably *marmoratis,* but possibly *grandis.*—Wilford A. Dence, *New York State College of Forestry, Syracuse, New York.*

## *SARCOCYSTIS RILEYI* (STILES, 1893) IN THE DOMESTIC FOWL, *GALLUS GALLUS*

*Sarcocystis* sp. has been reported from twenty-one species of birds, eleven in the United States (Erickson, 1940, Auk **57**: 514). However there have been few reports of its presence in the domestic fowl, *Gallus gallus.* The records in domestic fowl are: *Sarcocystis* sp. Stiles, 1893 (U. S. Dept. Agric. Bur. Animal Industry, Bull. **3**: 79–88) and Stiles, 1894 (Vet. Mag. Phila. **1**: 728), both in the United States; *Sarcocystis howarthi* v. Ratz, 1908 (Allattani Kozlemenyek **7**: 177) in Hungary; and *Sarcocystis gallinarum* Krause and Goranoff, 1933 (Z. Infektionskr. und Haustiere **43**: 261–279) in Bulgaria.

Recently we noted the presence of Sarcosporida in domestic fowl from North Adams, Michigan. The bird was submitted to the poultry clinic of this institution for diagnosis. Sarcocysts were noted on the external surface in the muscles of each thigh. Sections revealed a light infection in several muscle bundles. This specimen did not differ sufficiently from those of *Sarcocystis rileyi* from the mallard (*Anas platyrhynchos platyrhynchos*), the domestic mallard (*Anas platyrhynchos domestica*), or the black duck (*Anas rubripes*) in our possession to justify a new species.

It is apparent that *S. howarthi* v. Ratz, 1908, and *S. gallinarum* Krause and Goranoff, 1933, are synonymous with *S. rileyi* (Stiles, 1893).—Philip A. Hawkins, *Department of Bacteriology and Hygiene, Michigan State College.*

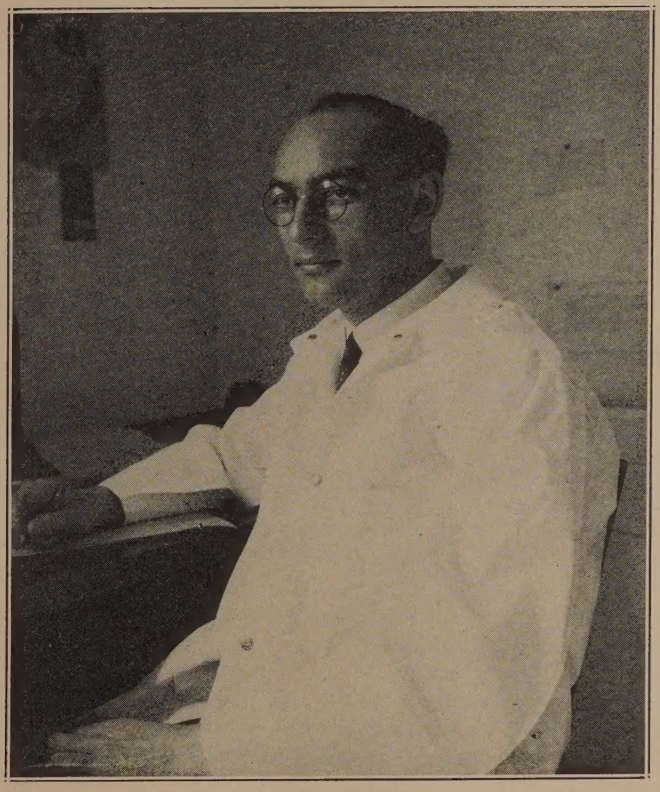
## THE ORIENTAL RAT FLEA (*XENOPSYLLA CHEOPIS*) IN MICHIGAN

On November 14, 1942, a wild house-rat (*Rattus norvegicus*) was killed in the animal house of the Zoology Department of the University of Michigan. Approximately one hundred specimens of the Oriental rat flea (*Xenopsylla cheopis*) were recovered.

Since 1925 when Wallace first reported this flea in Indianapolis, this medically important insect has been found in many of the central states including Indiana, Illinois, and Ohio. I have been unable to find a report of *Xenopsylla* from Michigan and consequently thought it advisable to record its expanding range.—Leo Jachowski, Jr., *Zoology Department, University of Michigan.*

## IN MEMORIAM

### William Albert Hoffman (1894–1943)



Dr. William A. Hoffman, Head of the Department of Medical Zoology, School of Tropical Medicine, San Juan, Puerto Rico, died on April 5, 1943. His death was a great shock to all his friends, for it ended prematurely an active and useful life which was remarkable for the overcoming of handicaps resulting from an attack of poliomyelitis at an early age.

Dr. Hoffman was born in Long Branch, New Jersey, on April 18, 1894. He received his B.S. from Cornell University in 1917 and his Sc.D. from the Johns Hopkins School of Hygiene and Public Health in 1924. The following year he was medical entomologist of the Haiti Survey for the International Health Division of

the Rockefeller Foundation, and in 1926 he joined the staff of the School of Tropical Medicine in Puerto Rico as parasitologist, a position which he held until his death.

At the Johns Hopkins University he was an assistant of the late Dr. F. M. Root, under whose guidance he completed his thesis for the doctorate on the *Culicoides* of North and Central America and the West Indies. This review included descriptions of 21 species, 6 of them new, and in addition, two new varieties. Primarily an entomologist, Dr. Hoffman maintained a deep interest in these insects, and came to be recognized as a leading authority on the biting midges in the New World. He continued to describe new species, but most of his efforts in this field were devoted to a cooperative project with Dr. Root on a monograph of the American *Culicoides*. Interrupted by Dr. Root's death, this work nevertheless resulted in a revision of the North American species of *Culicoides*, which included descriptions of 13 new species. At the time of his death, Dr. Hoffman was in possession of a considerable amount of unpublished data on biting midges and a large collection. Fortunately, his collection will be placed in the U. S. National Museum, where it will be available for further study. He was also well acquainted with the mosquitoes and had made several surveys in the Caribbean region.

His interests by no means were limited to entomology. His contact with tropical diseases led to many other investigations. In a series of papers written in part with Dr. E. C. Faust and others, on schistosomiasis, the distribution of *Schistosoma mansoni* in Puerto Rico was established, the intermediate host determined, and details of biology described. A sedimentation-concentration method for diagnosis of infections was devised, the use of which was urged as a means of preventing the introduction of the parasite into regions where it is not yet present. Unpublished results of his investigations on schistosomiasis included observations on pathology, and on the epidemiology and methods of control in Puerto Rico. Several papers were also written on *Fasciola hepatica*. The intermediate host was found and a precipitin test was prepared for diagnosing infections with this parasite. Finally, a survey of filariasis in Puerto Rico showed the incidence and distribution of *Wuchereria bancrofti* in the island.

Dr. Hoffman could walk only with the aid of special braces. In spite of this difficulty, in strenuous field trips he could set a stiff pace for many a younger and healthy collector by his enthusiasm and energy. He would refuse, in fact, resent any offer of aid, and his determination to equal or surpass the endeavors of people more favored physically, combined with his careful observations and sound reasoning, made him the successful scientist he aspired to be. Psychologically, he fought an uphill battle all his life. His deep love for fine music was a tremendous aid in this fight; many times he remarked that in periods of depression he could change his mood by playing his beloved violin or viola.

Parasitologists and entomologists will remember him through his contributions to their fields; his more intimate associates will remember him also as a kind and loyal friend.—L. E. Rozeboom, *School of Hygiene and Public Health, Johns Hopkins University.*